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SOME PROPERTIES OF A PHOSPHATASE FROM BOVINE SPLEEN

JOHN GLOMSET AND JERKER PORATH

Institute of Medical Chemistry and Institute of Biochemistry, Uppsala (Sweden)

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SUMMARY

The spleen phosphatase purified by previously described methods has been shown to be homogeneous by a number of criteria. Data have been presented concerning the sedimentation, diffusion, and the electrophoretic mobility of the purified enzyme. Preliminary calculations indicate that the mol. wt. of the enzyme is approximately 23,000. The absorption curve of the enzyme has been shown to have maxima at 280 mμ and 550 mμ as well as a shoulder in the 320 mμ region. It has not been possible to demonstrate the presence of any metals in the enzyme preparation. However, it has been demonstrated that the enzyme is a glycoprotein.

INTRODUCTION

Recently, the so-called "phosphoprotein phosphatase" of bovine spleen has been demonstrated to possess a number of very interesting properties[1-5]. The enzyme is activated by sulfhydryl compounds and inactivated by metal chelators. In addition the specificity of the enzyme appears to transect the borders of conventional phosphatase classification[6], introducing a new problem of nomenclature. Thus, highly purified "phosphoprotein phosphatase" is not only able to remove completely the phosphorus of α-casein, but is also a potent pyrophosphatase. Moreover, while having only slight activity toward glycerylphosphate and other low mol. wt. aliphatic phosphate monoesters, it is nevertheless highly active toward phenylphosphate and related compounds. Finally, an additional interesting attribute of the enzyme may be mentioned: as prepared from extracts of autolyzed spleen, it seems to be associated with a violet chromophore of unknown character.

In a previous publication[4], methods were described by which crude enzyme prepared by the method of SINGER AND FRUTON[2] could be further purified, yielding a basic protein of relatively low mol. wt. The purified enzyme moved as a single peak on zone electrophoresis in HAc–NaAc buffer, pH 5.6. It was in connection with these experiments that the violet color of the enzyme was first revealed.

The present paper is part of a more extensive investigation of the enzyme and is concerned with some of its physical and chemical properties.

METHODS

Preparation of the enzyme

The enzyme was prepared using essentially the same methods as previously described, except that vertical column electrophoresis[7] was employed instead of the horizontal block technique. Pevikon was again used as supporting medium[8,4]. As purchased commercially this polyvinylchloride–polyvinylacetate copolymer can be used directly with buffers having a pH in the vicinity of neutrality. However, when used in 1 M HAc (see below), it was found necessary to first wash the material thoroughly with glacial acetic acid in order to remove material absorbing in the ultraviolet.

Enzyme activity

Measurements of enzyme activity during the fractionation were performed by the incubation of 10 μl–50 μl aliquots of enzyme solution at 0° using *p*-nitrophenylphosphate ("Sigma 104") as substrate, mercaptoethanol (Merck) as activator, and sodium cacodylate (B.D.H.)–HCl, pH 6.0 as buffer in the final concentrations of 0.01 M, 0.02 M, and 0.05 M, respectively. The reaction was stopped by the addition of 9 volumes 0.1 M NaOH and the yellow color of the liberated *p*-nitrophenol measured in a Beckman B spectrophotometer at 400 mμ in 1 cm corex cuvettes[9]. It is necessary to exercise caution when measuring enzyme activity toward the final stages of purification. In the absence of more acidic proteins, the enzyme, which is itself strongly basic, is particularly liable to become adsorbed to the walls of pyrex test tubes, *etc.* Errors caused by adsorption may be reduced by coating test tubes, pipettes, *etc.* with silicon oil before use.

For convenience 1 unit of enzyme activity was taken to be the amount of enzyme required to liberate 1 μmole *p*-nitrophenol per ml incubation medium per min under the above conditions. Specific activity is defined as enzyme activity per mg protein N, as determined by the micro-Kjeldahl method.

Zone electrophoresis

The vertical bed of Pevikon used in the zone electrophoresis experiments with the purified enzyme was prepared as follows: the Pevikon powder was first passed through a 170 mesh screen. Subsequently it was thoroughly washed with glacial acetic acid on a Buchner funnel until the filtrate no longer yielded a precipitate or became opalescent on dilution with distilled water. Excess glacial acetic acid was then removed and the powder was suspended in 1 M HAc–0.05 M KCl. After sedimentation in order to remove the smaller particles the Pevikon was poured as a thick slurry into a 2.5 × 50 cm column provided with a bottom of thick filter paper. The Pevikon bed was washed with 1 l of 1 M HAc–0.05 M KCl and the absence in the eluate of impurities absorbing at 280 mμ was noted. Finally, 1 ml of N-dinitrophenylethanolamine solution was passed through the column in order to determine whether the column had been evenly packed. The column was then ready for use.

Optical density measurements

These measurements were performed in a Beckman DU spectrophotometer at 26°. The apparatus was equipped with a special housing unit allowing the use of 5-cm microcuvettes[10].

RESULTS

Electrophoretic properties

Moving boundary electrophoresis was performed in three different buffer systems with the Tiselius-Svensson electrophoresis apparatus, type LKB 3021 B. The measurements were performed with 0.4% solutions at 0.5°. In every instance only one migrating boundary could be observed with the Schlieren optical method of Philpot-Svensson. Moreover, the boundaries coincided in position with the boundary between the colored and noncolored solutions. The results of the measurements are compiled in Table I.

TABLE I

MOVING BOUNDARY ELECTROPHORESIS OF THE PURIFIED ENZYME

Buffer	*pH*	*Conductivity* Ω^{-1} *cm*$^{-1}$	*Current density* *A cm*$^{-2}$	*Mobility* *cm*^{2} *V*$^{-1}$ *sec*$^{-1}$
1 *M* acetic acid + 0.025 *M* KCl	2.4	$2.20 \cdot 10^{-3}$	$19.3 \cdot 10^{-3}$	$11.8 \cdot 10^{-5}$
0.05 *M* sodium cacodylate–HCl	6.0	$2.37 \cdot 10^{-3}$	$19.3 \cdot 10^{-3}$	$6.7 \cdot 10^{-5}$
0.05 *M* Tris–HCl	8.1	$1.33 \cdot 10^{-3}$	$16.3 \cdot 10^{-3}$	$5.0 \cdot 10^{-5}$

Measurements were not carried out at higher pH because of the irreversible inactivation and precipitation of the enzyme which occurs under more basic conditions (see later). The measurements nevertheless provide evidence of the homogeneity of the enzyme.

A vertical zone electrophoresis column containing Pevikon as supporting medium was prepared as described in METHODS. The height of the bed was 42 cm, and the bed contained 2.35 ml 1 *M* HAc–0.05 *M* KCl buffer per cm. 1.0 ml enzyme solution containing 14.3 mg protein was filtered into the column, followed by 2 ml of buffer. The column was brought into contact with electrode vessels containing approximately 7 l of buffer each, and the cooling jacket of the column was connected to a tap water source (12°). A current of 30 mA was then applied. Migration of the violet enzyme could be followed visually throughout the experiment. After 21 h the current was discontinued, the column was disconnected, and its liquid contents displaced in 2-ml fractions with fresh buffer into the tubes of a fraction collector. Determinations of absorption at 280 mμ and 540 mμ (the approximate absorption maximum of the enzyme in 1 *M* HAc–0.05 *M* KCl), and of enzyme activity were performed for each tube. The resulting electropherogram is shown in Fig. 1.

All parameter values are proportional throughout the entire enzyme zone within the limits of experimental error. This may be taken as a criterion of homogeneity.

Preliminary studies of the sedimentation and diffusion properties of the enzyme

These centrifugation studies were performed in a Spinco Analytical Centrifuge, Model E with an Anal. D-rotor. The sedimentation analysis was performed in 0.1 *M* NaAc–HAc buffer, pH 5.6 with a layer of 0.7% protein solution 12.0 mm in height. The speed of rotation was 59,780 rev./min. The temperature varied between 19.9° and 20.3° during the experiment. Only one sedimenting boundary was observed. See Fig. 2. The sedimentation constant at this concentration was $2.4 \cdot 10^{-13}$ S.

Diffusion at the above protein concentration, pH, and temperature (20.0°) in 0.1 *M* NaAc–HAc buffer, pH 5.6 was determined in the Tiselius-Svensson electrophoresis apparatus (Type LKB 3021 B). Determinations using the area method[11] yielded a value of $9.18 \cdot 10^{7}$ cm^2/sec and using the bredth of the diffusion curve at the inflexion points a value of $9.2 \cdot 10^{-7}$ cm^2/sec. The close similarity between the

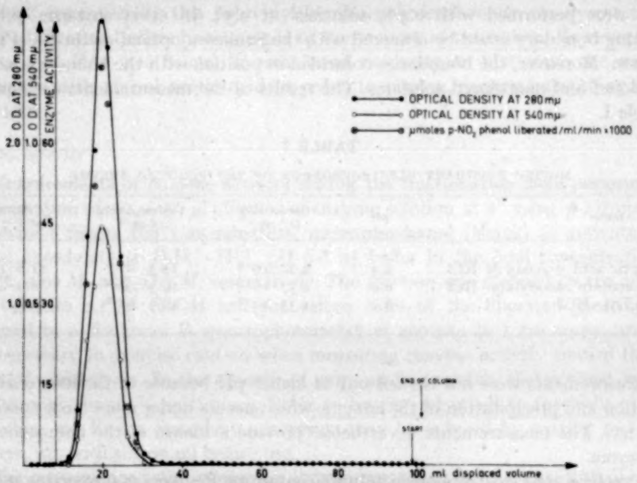

Fig. 1. Zone electropherogram of purified enzyme on a Pevikon column in 1 *M* HAc–0.05 *M* KCl. For further details see text.

Fig. 2. Sedimentation diagrams taken at 54 (a), 152 (b), and 214 (c) min after starting the experiment. For details see text.

two values can be considered a criterion of purity. If $1 - \bar{V}\varrho$ in the Svedberg formula is provisionally taken as equal to 0.25 an approx. value of 25,000 is obtained for the mol. wt. of the enzyme.

Determination of mol. wt. by the method of ARCHIBALD[12] was also performed. The cell was filled to 12 mm height with a 1% protein solution in 0.1 *M* NaAc–HAc, pH 5.6. The experiment was performed at 20° at an average of 11,530 rev./min. Taking $1 - \bar{V}\varrho$ as equal to 0.25, the following mol. wt. values for meniscus and bottom were obtained. See Table II.

In the case of a homogeneous material all values should be equal. In a polydisperse system the value at the bottom should increase while the value of the

TABLE II

DETERMINATION OF MOL. WT. ACCORDING TO ARCHIBALD

Time in min after start of expt.	*Mol. wt.*	
	at meniscus	*at bottom*
39	23,600	21,600
64	23,600	22,300
96	22,500	22,900
128	22,700	23,300
160	23,200	22,800
192	22,600	—
224	23,700	22,100
256	24,700	22,300
288	23,300	22,300
320	25,700	22,200
352	25,500	22,400
Average	23,700	22,700

meniscus should decrease (if $1 - V\varrho > 0$ as is the case here). The fact that the average mol. wt. at the meniscus is greater than that at the bottom is therefore indicative of a technical defect in measurement. This type of defect might possibly have been avoided had the method of SCHACHMAN[13] been employed, layering the protein solution over silicon oil. When these experiments were performed, however, we did not have this type of oil at our disposal. Nevertheless, the near agreement between the values can be considered as a rather good indication of homogeneity.

Optical properties of the purified enzyme

The optical density of the purified enzyme was studied in the region between 240 mμ and 800 mμ. Active preparations dissolved in 0.1 *M* HAc–NaAc buffer, pH 5.6 or sodium cacodylate–HCl buffer, pH 6.0 showed in addition to the expected 280 mμ maximum a second, broad maximum at about 550 mμ. Moreover, there was also a definite shoulder in the region of 310–330 mμ. See Fig. 3. Regardless of whether the enzyme had been subjected to heat treatment at 70° or not during the course of the preparation, the quotient of the 280 mμ/550 mμ readings of the purified enzyme had the same mean value of 3.39. An enzyme solution in 0.1 *M* cacodylate buffer, pH 6.0 having an optical density of 1.00 E_{280} was calculated to contain 0.105 mg N/ml.

It was of interest to determine the effect of pH on the optical properties of the enzyme as a step not only toward identification of the unusual chromophore group(s), but also toward establishing a possible correlation between activity and the optical properties of the enzyme. Some preliminary experiments were first performed in order to determine the effect of pH on the stability of the enzyme. In these experiments the enzyme was preincubated for 16 h at 37° in 0.1 *M* buffers of varying pH ranging from 1 to 9. After subsequent readjustment to pH 6.0 the enzyme activity was determined. The results of the activity determinations indicated that the enzyme is essentially stable under the above conditions in the pH interval between 2.0 to 8.0 in the absence of mercaptoethanol. At pH 1.0 and 9.0, however, irreversible inactivation of the enzyme is marked (HCl–KCl and Tris–HCl buffer, respectively). Moreover, the presence of 0.02 *M* mercaptoethanol during preincubation causes a decrease in enzyme activity at pH 7.0 and above, the inactivation at pH 8.0 being essentially complete. (In these experiments the presence of mercaptoethanol at pH 6.0 and below most frequently caused an increase in enzyme activity over the controls).

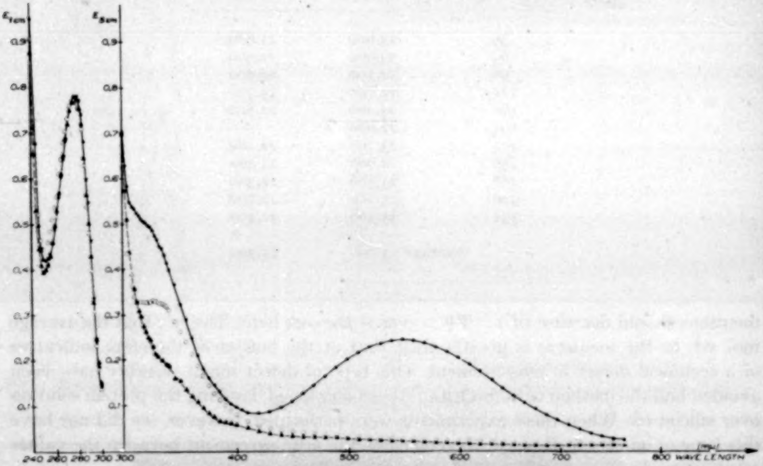

Fig. 3. Optical density curves of purified enzyme in 0.1 *M* cacodylate buffer, pH 6.0 (●); 0.1 *M* HCl–KCl buffer, pH 1.0 (○); and in 0.1 *M* cacodylate buffer, pH 6.0 after preincubation for 10 min at room temperature in 0.1 *M* HCl–KCl buffer, pH 1.0 (▲).

Finally, it was noted that at pH 8.0 (Tris–HCl) in the presence of mercaptoethanol extensive precipitation of the enzyme occurred.

The pH effect noted here is rather similar to that noted by **Sundararajan and Sarma**[1] and **Hoffman**[3] for their phosphatase preparations, particulary with regard to the effect of pH levels above 6. However, the present enzyme appears to be considerably more stable at lower pH levels than the preparation of **Hoffman**[3] (**Sundararajan and Sarma** have not recorded the effect of pH < 5 on their enzyme).

With the results of the above pH stability experiments in mind, the optical density of the enzyme at pH 1.0 was studied (0.1 *M* KCl–HCl buffer). The disappearance of the violet color of the enzyme stock solution was easily visible to the naked eye within a few min after the enzyme had been adjusted to pH 1.0 at room temperature. Subsequent optical density measurements (see Fig. 3) showed that while the 280 mμ absorption peak had not been significantly altered, the broad 550 mμ peak had disappeared. In addition it is of interest to note that while the shoulder at 310–330 mμ was still present, the absorption in this region had also decreased. In order to determine whether the disappearance of the 550 mμ peak was the result of a simple indicator-type effect, a solution of the enzyme which had first been adjusted to pH 1.0 was subsequently readjusted to pH 6.0 with 0.1 *M* cacodylate. Optical density measurements (also shown in Fig. 3) indicated the continued absence of the 550 mμ absorption peak. Moreover, a further drop in the absorption readings in the region immediately above 300 mμ had occurred.

Some analytical data

Analyses were performed on samples which after lyophilization had been washed with ethanol, acetone, and finally dried at room temperature in the weighing room. Nitrogen was determined by the micro-Kjeldahl technique and sulfur using a methylene blue method[14]. Copper and zinc were analyzed by the dithizone-carbontetrachloride method according to SANDELL[15]. Cobalt was analyzed according to DEAN[16]. The following analytical values were obtained after correction for moisture content: $N = 15.8\,\%$; $S = 0.84\,\%$; $Cu < 0.003\,\%$; $Zn < 0.012\,\%$; $Co < 0.005\,\%$.

A sample of approx. 4 mg was analyzed by spectrography using a direct current arc between graphite electrodes in an argon atmosphere. No trace of metals could be demonstrated in the spectrogram. The absence of paramagnetic metal ions was confirmed by electron spin resonance measurement at full sensitivity using a Varian V-4500 EPR spectrophotometer.

Analyses for hexose, hexosamine, and sialic acid were carried out by the methods of VASSEUR[17], ELSON AND MORGAN as modified by BLIX[18], and by the method of SVENNERHOLM[19], respectively. The following values were obtained after correction for moisture: hexose 3.21 %, hexosamine 1.45 %, and sialic acid < 0.1 %.

DISCUSSION

The enzyme behaves as a homogeneous substance under the conditions of electrophoresis and ultracentrifugation employed here. The limited availability of material unfortunately did not permit a determination of the partial specific volume making it necessary to postpone to the future a more exact determination of the mol. wt. from ultracentrifuge data. The presumed value of $1 - V\varrho = 0.25$ is probably somewhat too small. It should, however, be pointed out that the analytical data from the hexose and hexosamine determinations also support a value of approximately 23,000 for the mol. wt. If one mole of enzyme contains 4 hexose residues a mol. wt. of approximately 22,400 would obtain. If each mole of protein contained 2 hexosamine residues the mol. wt. would be approximately 24,700.

The physical characteristics which have been presented here should make it possible to determine more closely the relationship between the present phosphatase and bovine spleen phosphoprotein phosphatase as prepared by the method of SUNDARARAJAN AND SARMA[1]. Attention has already been called to some points of similarity and difference[4]. In view of the relatively low mol. wt. of the present enzyme the possibility remains that the autolysis step employed may have resulted in the cleavage of a larger original molecule, causing a significant change in the physical properties of the latter, but with the retention of the catalytically active part. If so it is noteworthy that evidently a single catalytically active molecule has resulted and not —apparently—a family of active fragments. Another possibility, pointed out previously[4], is that this small, highly basic enzyme is bound electrostatically to a larger, acidic protein or nucleic acid in the cell and that it is the latter which is fragmented by the autolysis procedure.

The nature of the violet chromophore is at present unknown. The possibility that it might be due to the presence of a strongly bound metal may be considered to be ruled out by the analyses performed here. The mechanism of the inhibition of the enzyme activity by chelators[5, 20] must also be sought elsewhere. Evidence will be presented later that the enzyme is activated by low concentrations of extraneous metals in a manner roughly similar to liver phosphoprotein phosphatase[21].

A question of considerable interest is the relation between the violet chromophore and the active center of the enzyme. The decrease in absorption at 550 mμ and 320 mμ on irreversible inactivation of the enzyme would tend to implicate the group(s) responsible for the absorption in these regions, although much more work remains to be done in order to prove this possibility. It can be mentioned, however, that activation of the enzyme with mercaptoethanol causes a slight shift in the 550 mμ peak toward lower wave lengths[20] at the same time that an immediate decrease in the absorption at 320 mμ seems to occur. Further work on this important problem is in progress.

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THE INFLUENCE OF GROWTH SUBSTRATES ON METABOLIC PATHWAYS IN *MICROCOCCUS DENITRIFICANS*

H. L. KORNBERG, J. F. COLLINS* AND (in part) D. BIGLEY**

M.R.C. Cell Metabolism Research Unit, Department of Biochemistry, and
Dyson Perrins Laboratory, Department of Chemistry,
University of Oxford, Oxford (Great Britain)

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SUMMARY

1. Autotrophically grown *M. denitrificans* incorporated ^{14}C from $^{14}CO_2$ initially into phosphorylated compounds, the chief component of this fraction being 3-phosphoglyceric acid.

2. Extracts of such cells catalysed the incorporation of isotope from $NaH^{14}CO_3$ into 3-phosphoglyceric acid, in the presence of ribulose 1,5-diphosphate, or of ribose 5-phosphate + ATP.

3. These results show that autotrophic growth of the organism involves the CALVIN cycle of assimilation of carbon dioxide.

4. Extracts of the organisms grown on acetate did not catalyse the incorporations of isotope from $NaH^{14}CO_3$ described under 2 above, indicating that the CALVIN cycle did not operate under these conditions.

5. Extracts of *M. denitrificans* grown autotrophically or on a variety of carbon substrates differed in their ability to effect the component reactions of the glyoxylate cycle. Whilst all extracts were qualitatively shown to contain citrate condensing enzyme and malate synthetase, only those from acetate-grown cells contained high isocitratase activity. The activity of glyoxylate cycle enzymes in acetate-grown cells was sufficiently high to account for the growth of *M. denitrificans* on acetate as sole carbon source.

6. These findings show that change in the mode of growth, from autotrophy to growth on acetate, was accompanied by an alteration of metabolic pathways from CALVIN cycle to glyoxylate cycle, and suggest that isocitratase is under adaptive control.

7. Studies on the effect of other carbon sources on the isocitratase formed by acetate-grown cells indicate that they control both the activity and formation of the enzyme by feed-back mechanisms.

8. It is suggested that such feed-back mechanisms control the intracellular operation of the glyoxylate cycle.

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* Present address: National Institute for Medical Research, Mill Hill, London, N.W.7.
** Present address: Dept. of Chemistry, Purdue University, Lafayette, Ind. (U.S.A.).

INTRODUCTION

Micrococcus denitrificans[1] grows readily under autotrophic conditions in a mineral salts medium gassed with H_2, O_2 and CO_2, and also heterotrophically on a variety of substrates, including acetate. A number of autotrophic micro-organisms have been shown to assimilate carbon from carbon dioxide by a pathway involving as a first step cleavage of ribulose 1,5-diphosphate by CO_2 to yield 3-phosphoglyceric acid, catalysed by carboxydismutase[2] (for review, see [3,4]). This enzyme has been found to be present only in micro-organisms grown autotrophically, or on formate[5,6] or pentoses[7] and to be absent from such organisms grown on oxalate[6], glucose[7], or lactate[8]. Similarly, studies on *Micrococcus lysodeikticus*[9] and on *Pseudomonas ovalis* Chester[10,11] have shown that a key enzyme of the glyoxylate cycle[12,13], isocitratase, is present in high concentration only after growth on acetate or precursors of acetate. It was of interest, therefore, to study in one species of micro-organism the changes in metabolic pathways concomitant with change of carbon source, from carbon dioxide to acetate. It is shown in this paper that, when carbon dioxide is the sole carbon source, the biosynthesis of cell constituents by *M. denitrificans* involves the CALVIN cycle[3], but when acetate is the carbon source it involves the glyoxylate cycle[12,13]. The change from autotrophy to growth on acetate is accompanied by a disappearance of carboxydismutase and the appearance of isocitratase. Evidence is also presented which bears on the cellular regulation of the operation of the glyoxylate cycle.

METHODS

Maintenance and culture of the organisms

The original culture of *Micrococcus denitrificans* used was a gift from Dr. J. LASCELLES. Organisms were maintained by serial subculture, at fortnightly intervals, on agar slopes containing per 100 ml of medium: Evans' peptone, 1 g; glycerol, 0.8 ml; K_2HPO_4, 100 mg; $MgSO_4$, 25 mg; agar agar, 2 g; and water to 100 ml. The medium used for culture of the organisms under autotrophic conditions (A) contained K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2HPO_4$, 0.8 g; $CaCl_2 \cdot 6H_2O$, 0.04 g; KNO_3, 20.2 g; and water to 500 ml. This solution was sterilized at 15 lbs./in^2 for 15 min and was mixed with 10 ml of sterile 5% sodium bicarbonate, 10 ml of sterile $FeSO_4$ (containing 10 mg of $FeSO_4$ in 0.1 N HCl) and 480 ml of sterile water. The combined medium was placed in a flask attached to a closed gas circulating system. From a reservoir (capacity: 20 l) a mixture of 10% CO_2, 30% air and 60% H_2 was passed through a sterile cotton wool filter into this vessel, the gas mixture entering through a sintered disc at the bottom. The gas was returned to the reservoir through a second sterile filter. The organisms were grown at 25°.

The medium used for culture of the organisms under heterotrophic conditions (B) has been previously described[14]; yeast extract and carbon sources other than acetate were added where indicated in the text.

Incorporation of $^{14}CO_2$ by M. denitrificans *growing autotrophically*

Cells, harvested from medium (A) during the logarithmic phase of growth, were suspended in 10 mM phosphate buffer, pH 6.9, at a concentration of 1 mg dry wt./ml. The suspension was gassed with a mixture of 93% H_2, 6% O_2 and 1% CO_2 for

30 min, to bring the cells into a steady state of growth. The suspension was then flushed vigorously with a mixture of 98 % H_2 and 2 % O_2, for 30 sec, and a portion thereof (20 ml) sucked into a 50-ml glass syringe, which contained an equilibrated mixture of 1 ml of $Na_2{}^{14}CO_3$ (100 μC; 100 μmoles), 5 ml of 10 m*M* phosphate buffer, pH 6.9, and 10 ml of the gas mixture (98 % H_2 and 2 % O_2). The syringe was sealed by plunging the needle into a rubber bung and was shaken vigorously. Samples (5 ml) were squirted into tubes containing 20 ml of cold methanol at known times. These 80 % methanolic suspensions were boiled for 5 min and measured portions thereof plated on aluminium planchettes which, after acidification to remove residual carbon dioxide, were assayed for radioactivity with a thin-window G.M. β-counter tube.

The remaining portions of the 80 % methanolic suspensions were centrifuged, and the precipitated materials extracted with 5 ml of boiling 20 % methanol. The supernatant fractions of both extractions were combined, concentrated, and analysed by two-dimensional chromatography and autoradiography as previously described[14].

Preparation of extracts

Extracts of bacteria were prepared either by disintegration of frozen cells in the Hughes press[15] or by subjecting suspensions of the organisms in 0.9 % KCl for 3 min to the output of a 600 W Mullard magnetostrictor oscillator (25 kc, 3.5 A). The suspensions of disintegrated bacteria obtained by either technique were centrifuged at 25,000 × *g* in an International refrigerated centrifuge at 0°; the supernatant solutions were used for experimental purposes as described in the text.

Incorporation of $^{14}CO_2$ by cell extracts

Crude extracts of cells grown autotrophically or on acetate and crushed in a Hughes press[15], were suspended in 9 vol. of 0.1 *M* potassium phosphate, pH 7.2, and were incubated for 30 min at 22° with one hundredth volume of a mixture of 0.1 % (w/v) ribonuclease and deoxyribonuclease (L. Light & Co., Colnbrook, Bucks., Engl.). After centrifugation, the clear supernatant solutions (containing the extract respectively of 25 mg or 11.9 mg dry wt. of crushed cells/ml) were used for experimental purposes.

The complete system contained, in 0.5 ml: 5 μmoles of potassium phosphate, pH 7.4; 2.5 μmoles of $MgCl_2$; 1.25 μmoles of $Na_2{}^{14}CO_3$ (5 μC); 0.05 ml of cell extract; other substrates as indicated; and water. The system was incubated at 30° for 15 min, and the reaction stopped by addition of 0.5 ml of 95 % ethanol. The precipitate, removed by centrifugation, was washed with 0.5 ml of 20 % aqueous ethanol. The combined supernatant solutions were evaporated to small known volumes at 60° under a stream of nitrogen, and samples thereof were applied to the origin positions of paper chromatograms. This material was acidified with 2 *N* acetic acid, and its radioactivity measured by direct assay with a thin end-window β counter tube (G.E.C. type 2 B 2). In one case, in which the radioactivity incorporated was sufficiently high to warrant further analysis (see Table II, tube III), the sample was analysed by two-dimensional chromatography and autoradiography[14].

Special chemicals and other procedures used

$Na_2{}^{14}CO_3$ and $NaH^{14}CO_3$ were prepared from $Ba^{14}CO_3$ by acidification of the $Ba^{14}CO_3$ and quantitative absorption of the evolved $^{14}CO_2$ in standard NaOH. The solution of [1-^{14}C]acetate used was prepared[14] from sodium [1-^{14}C]acetate. All isotopic materials were purchased from the Radiochemical Centre, Amersham, Bucks. Potassium L_s-isocitrate was a gift from Dr. H. B. VICKERY (Connecticut Agricultural Station, New Haven, Conn., U.S.A.). Samples of ribulose 1,5-diphosphate and 3-phosphoglyceric acid were gifts from Dr. J. R. QUAYLE. Other materials and enzymic procedures used were those previously described[10, 12, 14] unless stated otherwise in the text.

RESULTS

The incorporation of $^{14}CO_2$ by autotrophically-growing Micrococcus denitrificans

Measurement of the radioactivity of cells and cell fractions after incubation for brief periods (0.5–8 min) with $Na_2{}^{14}CO_3$ showed that both the methanol-soluble and methanol-insoluble fractions rapidly incorporated ^{14}C (Table I). Analysis of the distribution with time of isotope within the methanol-soluble fractions further showed that ^{14}C from $Na_2{}^{14}CO_3$ had initially entered phosphorylated materials and that other compounds (such as amino acids and intermediates of the tricarboxylic acid cycle) progressively acquired radioactivity thereafter (Fig. 1A). Further resolution of the constituents of the mixture of phosphorylated compounds showed that the chief labelled component thereof was 3-phosphoglyceric acid (Fig. 1B). This compound was identified by co-chromatography with authentic 3-phosphoglyceric acid, and by the formation of glyceric acid as the only labelled product after treatment with phosphatase ("Polidase S": Schwarz Laboratories Inc., Mount Vernon, N.Y., U.S.A.).

TABLE I

INCORPORATION OF ISOTOPE FROM $Na_2{}^{14}CO_3$ BY AUTOTROPHICALLY GROWING *Micrococcus denitrificans*

Cells which had been harvested from medium (A) (see METHODS) and pre-incubated with phosphate buffer, pH 6.9, and a gas mixture of 93 % H_2, 6 % O_2 and 1 % CO_2 for 30 min, were flushed for 30 sec with 98 % H_2 and 2 % O_2. Of this suspension, 20 ml (containing 20 mg dry wt. of cells) were sucked into a syringe containing 1 ml of $Na_2{}^{14}CO_3$ (100 µC, 100 µmoles, giving $6.5 \cdot 10^6$ counts/min under the conditions of radioassay used), 5 ml of phosphate buffer, pH 6.9 and 10 ml of a gas mixture of 98 % H_2 and 2 % O_2. Samples (5 ml) were squirted into tubes containing 20 ml of methanol at known times, after which the suspensions were boiled. Of these suspensions, known volumes were pipetted onto planchettes, acidified to remove residual $Na_2{}^{14}CO_3$, and their total radioactivity assayed with a β-G.M. counter tube. The radioactivity is expressed as counts/min corrected for background. The radioactivity in the methanol-soluble fractions was assayed in a similar manner by plating known volumes of the combined supernatant solutions, after extraction of the cells with 80 % and 20 % methanol.

Time (min)	*Radioactivity (counts/min × 10^{-4}) in*	
	Total cells	*Methanol-soluble fraction*
0.5	32.9	28.0
1.0	62.3	51.0
2.0	107	84.0
4.0	197	123
8.0	402	152

The high proportion of label incorporated initially (> 90 % of the total ^{14}C fixed in 0.5 min) in phosphorylated compounds, and the predominance of 3-phospho-

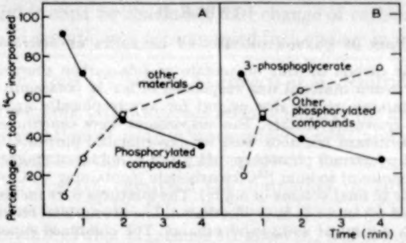

Fig. 1. Variation with time of the percentage distribution of ^{14}C from $^{14}CO_2$ incorporated into the constituents of the methanol-soluble fraction of *M. denitrificans* growing autotrophically. A: ●——● = Percentage incorporated into phosphorylated compounds; ○——○ = percentage incorporated into other materials (tricarboxylic acid cycle intermediates and amino acids). B: ●——● = Percentage of 3-phosphoglycerate present in the phosphorylated compounds; ○——○ = percentage of other phosphorylated materials (sugar phosphates).

glyceric acid (> 70 % in 0.5 min) in these phosphorylated compounds, indicate the operation, during the autotrophic growth of *Micrococcus denitrificans*, of the CALVIN cycle[3] of assimilation of carbon dioxide. This indication was strengthened by demonstration of a key enzyme of the CALVIN cycle[3], carboxydismutase[2], in high activity in extracts of *M. denitrificans* thus grown.

The incorporation of $^{14}CO_2$ by extracts of autotrophically-grown M. denitrificans

When extracts of autotrophically-grown cells were incubated with [^{14}C]bicarbonate (of activity $3.2 \cdot 10^5$ counts/min under the conditions of assay used), only traces of radioactive isotope were incorporated (Table II, tube I). Addition to this system of ribulose 1,5-diphosphate resulted in an over one-hundred fold increase in the incorporation of isotope (Table II, tube III). Analysis of the labelled material thus formed showed over 90 % of the radioactivity to have been incorporated into 3-phosphoglyceric acid. This shows that the extract contained high carboxydismutase activity, and supported the view that autotrophic growth of *M. denitrificans* involved the operation of the CALVIN cycle[3]. Replacement of ribulose 1,5-diphosphate by ribose-5-phosphate and ATP[*] (Table II, tube II) also led to a significant enhancement of isotope incorporation, of over 4 times the radioactivity in the absence of added substrates. This finding indicated the presence of the further two enzymes of the CALVIN cycle[3], pentose phosphate isomerase and ribulose phosphate kinase, in the extract.

The incorporation of $^{14}CO_2$ by extracts of acetate-grown M. denitrificans

Repetition of the above experiment with cell extracts obtained from *M. denitrificans* grown on acetate as sole carbon source gave entirely different results: neither the addition of ribulose 1,5-diphosphate (Table II, tube VI) nor of ribose 5-phosphate and ATP (Table II, tube V) enhanced the low incorporation of ^{14}C from $NaH^{14}CO_3$ observed in the absence of added substrates (Table II, tube IV). Carboxydismutase, and one or both of the other enzymes of the CALVIN cycle[3] demonstrated in extracts of autotrophically-grown cells, were thus absent from extracts of the same organism

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[*] The following abbreviations will be used: ATP = adenosine triphosphate; AMP = adenosine monophosphate; PP = pyrophosphate.

TABLE II

THE INCORPORATION OF CARBON DIOXIDE BY EXTRACTS OF *Micrococcus denitrificans*

Cells, grown on either carbon dioxide or acetate as sole carbon sources, were crushed in the HUGHES press[15]. The frozen material was suspended in 0.1 *M* potassium phosphate, pH 7.2, to give suspensions containing 25 mg dry wt./ml for autotrophically grown cells, and 11.9 mg dry wt./ml for those grown on acetate. The suspensions were centrifuged at 0° for 10 min at 25,000 g, and the supernatant fractions used for experimental purposes. The incubation system contained 5 μmoles of potassium phosphate, pH 7.4; 2.5 μmoles of magnesium chloride; 0.05 ml of cell extract; 1.25 μmoles of sodium [^{14}C]bicarbonate (containing 5 μC of ^{14}C); other substrates as indicated, and water to final volume of 0.5 ml. The mixtures were incubated for 15 min at 30°. To each tube was added 0.5 ml of 95% (v/v) ethanol. The precipitate, removed by centrifugation was washed once with 0.5 ml of 20% (v/v) ethanol. The combined supernatant solutions were evaporated to smaller volumes, applied to the origin positions of paper chromatograms and acidified with 2 *N* acetic acid. The radioactivity was measured by direct assay on the paper, with a thin end-window β counter tube (G.E.C. type 2 B 2).

Tube No.	*Carbon source for growth*	*Additional substrates*	*Radioactivity incorporated (counts/min)*
I	Carbon dioxide	None	113
II		2.5 μmoles of ribose-5-phosphate + 2.5 μmoles of ATP	475
III		0.5 μmole of ribulose-1,5-diphosphate	12,751*
IV	Acetate	None	157
V		2.5 μmoles of ribose-5-phosphate + 2.5 μmoles of ATP	190
VI		0.5 μmole of ribulose-1,5-diphosphate	175

* Chromatographic analysis[14] showed over 90% of the radioactivity to be incorporated into 3-phosphoglyceric acid.

TABLE III

THE INCORPORATION OF ^{14}C FROM [1-^{14}C]ACETATE BY EXTRACTS OF *Micrococcus denitrificans* GROWN AUTOTROPHICALLY OR ON ACETATE

Extracts of cells, which had been grown on either carbon dioxide or acetate as carbon source were those described under Table II. The incubation system contained 100 μmoles of potassium phosphate, pH 7.5; 10 μmoles of glutathione; 5 μmoles of $MgCl_2$; 0.09 μmoles of coenzyme A; 1.9 μmoles of sodium [1-^{14}C]acetate (containing 10 μC of ^{14}C); 10 μmoles of potassium acetate; 0.2 ml of cell extract; 5 μmoles of ATP; additional substrates as indicated and water to final volume of 1 ml. The mixtures were incubated for 30 min at 30°. To each was added 2 ml of hot 95% (v/v) ethanol, the precipitates were washed with 20% (v/v) ethanol and the combined supernatant solutions were analysed by chromatography and autoradiography[14].

Tube	*Carbon source for growth*	*Additional substrates*	*Labelled products formed (mμmoles)*			
			Succinate	*Citrate*	*Malate*	*Fumarate*
I	Carbon dioxide	None	1	2	0	2
II		10 μmoles of oxaloacetate	2	87	0	2
III		7 μmoles of glyoxylate	0	0	167	0
IV		20 μmoles of isocitrate (DL + Allo)	1	7	2	1
V	Acetate	None	17	7	9	6
VI		10 μmoles of oxaloacetate	13	783	0	0
VII		7 μmoles of glyoxylate	9	24	789	17
VIII		10 μmoles of L_s(+)-isocitrate	25	28	1259	0

grown on acetate, and it must be concluded that change of carbon source for growth, from carbon dioxide to acetate, was accompanied by a change in metabolic pathways.

The incorporation of ^{14}C from [^{14}C]acetate by extracts of M. denitrificans

This change in metabolic pathways was reflected also in the ability of cell extracts to catalyse the incorporation of isotope from added [1-^{14}C]acetate when incubated with substrates of enzymes of the glyoxylate cycle[12,13]. Extracts of cells grown either autotrophically or on acetate, catalysed the formation of labelled citrate, when incubated with oxaloacetate (Table III, tubes II and VI) and of labelled malate when incubated with glyoxylate (Table III, tubes III and VII), which indicated the presence in both extracts of condensing enzyme[16] and of malate synthetase[17]. The amounts of citrate and malate formed by the former extract were only 5–10 % of those by the latter. However, for reasons discussed below, these values are not necessarily true indications of the relative abundance of the two enzymes in the extracts.

A much greater relative difference was shown by the two types of extract in their ability to catalyse the formation of labelled malate from labelled acetate and isocitrate. Whilst the extract obtained from autotrophically-grown cells promoted the formation of 2 mμmoles of malate, that from acetate-grown cells catalysed the formation of 1259 mμmoles. Hence, on the basis of equivalent protein concentrations in the extracts, the activity in the latter of the rate-limiting step in the overall[12] reaction (I):

$$\text{isocitrate} + [1\text{-}^{14}\text{C}]\text{acetate} \longrightarrow [1\text{-}^{14}\text{C}]\text{malate} + \text{succinate} \qquad \text{(I)}$$

was at least a thousand times higher than that found in the former extract.

Acetic thiokinase in extracts of M. denitrificans

Since the overall reaction (I) is the sum[12] of the reactions leading from acetate to acetyl coenzyme A, catalysed by acetic thiokinase[18] (II):

$$\text{acetate} + \text{ATP} + \text{coenzyme A} \longrightarrow \text{acetyl coenzyme A} + \text{AMP} + \text{PP} \qquad \text{(II)}$$

leading from isocitrate to glyoxylate, catalysed by isocitratase[19–22] (III)

$$\text{isocitrate} \rightleftharpoons \text{succinate} + \text{glyoxylate} \qquad \text{(III)}$$

and leading to the formation of malate (IV) by interaction of the acetyl coenzyme A and glyoxylate formed in reactions (II) and (III), catalysed by malate synthetase[17]:

$$\text{acetyl coenzyme A} + \text{glyoxylate} \longrightarrow \text{malate } (+ \text{ coenzyme A}) \qquad \text{(IV)}$$

it was conceivable that any of these component reactions might impose a rate-limiting step in the overall formation of malate from acetate and isocitrate. Extracts of *M. denitrificans* grown on acetate as sole carbon source contained acetic thiokinase activity sufficient to catalyse the formation of 4.7 μmoles of acethydroxamic acid/mg of soluble protein/h in the test of JONES AND LIPMANN[18], the formation of the hydroxamate being dependent on the presence of added coenzyme A and of acetate (Fig. 2). Extracts of *M. denitrificans* grown on carbon dioxide, malate or glucose as carbon sources also contained demonstrable acetic thiokinase activity, but the enzymic activity of such extracts was variable and only 10–30 % of that found with acetate-grown cells. This consideration shows that the results quoted in Table III (tubes II, III, VI and VII) are of only qualitative significance, since the activation of acetate, via reaction (II), was one rate-limiting step. Hence this procedure does not

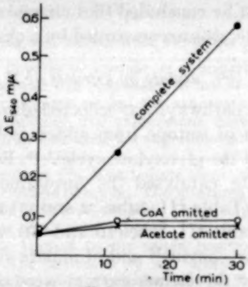


Fig. 2. Formation of acethydroxamic acid[18] from acetate by extracts of acetate-grown *M. denitrificans.*

permit of conclusions to be drawn on the relative abundance of condensing enzyme and malate synthetase in the extracts. However, since the amount of labelled malate formed from [^{14}C]acetate and glyoxylate by the extract of acetate-grown cells was only 5 times that formed by extracts from autotrophically-grown cells (Table III, tubes VII and III), it was clear that the six hundred-fold difference observed between the ability of the two extracts to effect the overall reaction (I) (Table III, tubes IV and VIII) could not be attributed to lack of either acetic thiokinase or of malate synthetase (reactions (II) and (IV)) in the autotrophically-grown cells, and suggested that, under these conditions, the levels of isocitratase (III) imposed a further and quantitatively more important rate-limiting step.

The incorporation of ^{14}C from [1-^{14}C]acetate by extracts of M. denitrificans *grown on other substrates*

The reduction in the ability of cell extracts from autotrophically grown *M. denitrificans* to catalyse the incorporation of isotope from [1-^{14}C]acetate when incubated with isocitrate (reaction (I)) noted in Table III was demonstrated also when extracts of cells grown on other substrates were examined. Extracts of cells grown on glucose or malate contained variable amounts of acetic thiokinase (reaction (II)) and thus differed in their ability to effect the activation of [1-^{14}C]acetate to [1-^{14}C]acetyl coenzyme A. However, the results obtained on incubating such extracts with [1-^{14}C]-acetate, ATP, coenzyme A and either glyoxylate (Table IV, tubes III and VII) or oxaloacetate (Table IV, tubes II and VI) showed qualitatively that both malate synthetase (reaction (IV)) and condensing enzyme were present but, as indicated by the low incorporation of isotope noted in the presence of isocitrate (Table IV, tubes IV and VIII) that the level of isocitratase in these extracts was considerably lower.

Repetition of these tests with an extract prepared from cells grown on 50 m*M* acetate + 0.05 % yeast extract (Table IV, tubes IX to XII) gave surprisingly similar results: again, both condensing enzyme and malate synthetase were present and isocitratase was largely absent. This finding was particularly striking since cells grown on this medium, but without added yeast extract (Table III, tube VIII) had, when incubated with isocitrate, catalysed an incorporation of isotope from [1-^{14}C]acetate 1.6 times greater than that noted on incubation of this extract with glyoxylate (Table III, tube VII). Since it was known that the added yeast extract contained

TABLE IV

THE INCORPORATION OF ^{14}C FROM [1-^{14}C]ACETATE BY EXTRACTS OF *Micrococcus denitrificans* GROWN ON OTHER SUBSTRATES

Cells were grown on medium (A) (see METHODS) with 50 mM acetate, malate or glucose as major carbon source. One of these growth media also contained "Difco" yeast extract to final concentration of 0.05 % (w/v). Extracts were prepared and assayed as described under Table III.

Tube	*Carbon source for growth*	*Additional substrates*	*Labelled products formed (mμmoles)*			
			Succinate	*Citrate*	*Malate*	*Fumarate*
I	Malate	None	2	8	5	6
II		Oxaloacetate	29	698	0	25
III		Glyoxylate	4	0	421	127
IV		L_s-isocitrate	5	10	10	12
V	Glucose	None	2	4	2	0
VI		Oxaloacetate	9	163	10	12
VII		Glyoxylate	4	8	121	39
VIII		L_s-isocitrate	4	6	2	0
IX	Acetate + yeast extract	None	2	0	1	2
X		Oxaloacetate	3	176	0	4
XI		Glyoxylate	3	3	131	30
XII		L_s-isocitrate	4	0	4	2

quantities of materials which might serve as alternative carbon sources (*e.g.* succinate), these findings indicated that the formation of isocitratase by the cells was profoundly influenced by the nature of the growth substrate, and that even small amounts of carbon sources other than acetate reduced considerably the intracellular levels of this enzyme. An investigation was therefore undertaken of some of the environmental factors affecting the formation of isocitratase by *M. denitrificans*.

The effect on acetate-grown cells of other growth substrates

When suspensions of *M. denitrificans*, which had been grown on acetate as sole carbon source, were allowed to continue to grow in fresh acetate-growth medium, isocitratase continued to be formed at a rate directly proportional to the increase in cellular mass (Fig. 3, line A). The plot of the enzyme content/ml of suspension against the cell density was linear: the line had a slope[23] of $P = 1.58$ and, on extrapolation, passed through the origin. This finding contrasted with those obtained when such acetate grown cells were placed in fresh growth media containing fumarate or L-malate as carbon sources. In these cases (Fig. 3, lines B or C) the lines obtained by plotting the enzyme content/ml of suspension against the cell density had slopes[23] of $P = 0.27$ and 0.23 respectively and, on extrapolation, did not pass through the origin. The slopes of the lines obtained under these conditions, termed by MONOD[23] "the differential rate of synthesis of the enzyme", are the ratio of increase in enzyme to the increase of bacterial mass. It therefore appeared that, for unit increase in cellular mass, isocitratase synthesis was reduced by 80 % in passing from acetate to fumarate or L-malate as carbon sources.

An even more striking reduction of isocitratase formation was observed when acetate-grown cells were placed in fresh growth medium containing succinate as sole carbon source. Whilst continued growth on acetate was accompanied by an un-

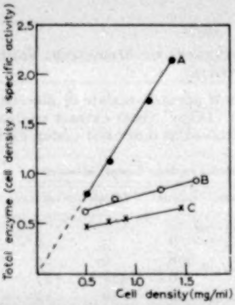


Fig. 3. Influence of carbon source on isocitratase formation by acetate-grown *M. denitrificans*. Suspensions of acetate-grown cells continued to grow in fresh growth media containing A: acetate (●——●); B: fumarate (○——○); or C: L-malate (×——×) as carbon source. The total enzyme content (cell density × specific activity) was calculated from the enzymic activity[10, 22] of sonic extracts of cells thus grown.

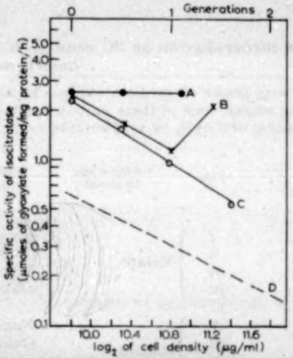


Fig. 4. Effect of succinate on isocitratase formation by acetate-grown *M. denitrificans*. Acetate grown cells continued to grow in media containing A: 75 m*M* acetate (●——●); B: 60 m*M* acetate + 15 m*M* succinate (×——×), or C: 75 m*M* succinate (⊙——⊙) as carbon sources. The specific activity (μmoles of glyoxylate formed at pH 6.0/mg soluble protein/h), determined from analysis of sonic extracts[10] is plotted against the $\log_2$ of the cell density. Line D is a plot of the calculated "diluting out" curve.

diminished rate of isocitratase formation and the specific activity of the enzyme remained high and constant (Fig. 4, line A), continued growth on succinate resulted in cessation of isocitratase formation. The specific activity of the enzyme under these growth conditions fell by 50 % for each increase in cell population of 0.9 of a generation (Fig. 4, line C), which decrease was slightly greater than would be expected were the enzyme "diluted out" (Fig. 4, line D) during growth. Acetate-grown cells placed in fresh growth medium containing both acetate and succinate, in the ratio 4:1 (Fig. 4, line B), also failed to continue to synthesise isocitratase for one generation and also manifested a decrease in specific activity of the enzyme slightly greater than the calculated "diluting out" curve (Fig. 4, line D). After the limited amount of succinate in this growth medium had been utilized, isocitratase formation recommenced. However, this renewed formation of the enzyme did not result in the maintenance of the specific activity of the isocitratase at the low intracellular level reached at the point of exhaustion of the succinate in the growth medium, but instead resulted in a rapid rise in the specific activity of the enzyme to a level approaching that in the cells which had not received succinate. The continued growth of the organisms on acetate was thus apparently accompanied by a formation of isocitratase relatively greater than the formation of other cell proteins.

This effect was also noted when succinate, to final concentration of 15 m*M*, was added at different times to acetate-grown cells continuing to grow in fresh media containing 75 m*M* acetate as sole carbon source. Whilst organisms continuing to grow on acetate continued to form isocitratase at the same rate as other cell proteins, and maintained the enzyme at constant specific activity (Fig. 5, line A), addition of succinate at various times (Fig. 5, curves B, C and D) resulted in immediate depression of the specific activity of the enzyme, the rate of fall increasing with increasing ratio

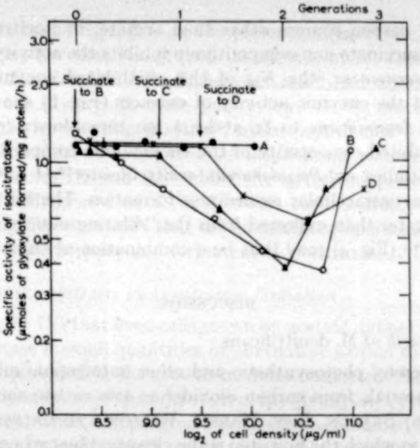


Fig. 5. Effect of succinate on isocitratase formation by acetate-grown *M. denitrificans*. All media contained 75 m*M* acetate. Succinate, to final concentration of 15 m*M*, was added to B (○——○) at zero time, to C (▲——▲) at 1 h and to D (×——×) at 2 h. Samples were removed and analysed as previously described[10].

of succinate: acetate in the medium. After the succinate had been utilized, the specific activity of the isocitratase rose sharply to the level obtaining in the cells before addition of succinate.

These results can be explained by a two-fold effect on isocitratase activity and content: (a) an inhibition by succinate of isocitratase activity, and (b) an inhibition

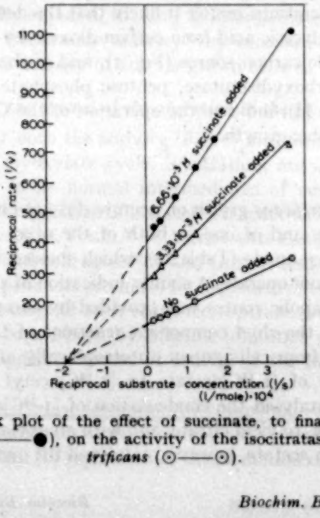


Fig. 6. LINEWEAVER-BURK plot of the effect of succinate, to final concentration of 3.3 m*M* (△——△) or 6.6 m*M* (●——●), on the activity of the isocitratase of acetate-grown *M. denitrificans* (⊙——⊙).

by succinate, and carbon sources other than acetate, of isocitratase formation. It was known[28] that succinate non-competitively inhibits the activity of the isocitratase isolated from *P. aeruginosa*, the K_m of the uninhibited enzyme being $5 \cdot 10^{-4}$ M. Kinetic analysis of the enzymic activity of extracts (Fig. 6) showed the K_m of the isocitratase of *M. denitrificans* to be at least ten times lower ($5 \cdot 10^{-5}$ moles/l), and that succinate inhibited the activity of the enzyme non-competitively, the K_i being $3 \cdot 10^{-3}$ moles/l. Studies on *Pseudomonas ovalis* Chester[31,32] have similarly shown succinate to repress intracellular isocitratase formation. The fall in specific activity of cell extracts greater than expected from the "diluting out" of the enzyme during growth on succinate (Fig. 4) may thus be a combination of these effects.

DISCUSSION

The autotrophic growth of M. denitrificans

The route whereby photosynthetic and other autotrophic micro-organisms synthesize cellular materials from carbon dioxide[4] as sole carbon source was first elucidated by BASSHAM, BENSON, KAY, HARRIS, WILSON AND CALVIN[24] and was shown to involve a cycle[3] in which the first step is the cleavage[2] by carbon dioxide of ribulose 1,5-diphosphate to two molecules of 3-phosphoglyceric acid. Subsequent transformations of 3-phosphoglyceric acid serve to form xylulose 5-phosphate which, in reactions catalysed by pentose phosphate isomerase[25] and ribulose phosphate kinase[26,27] regenerates the carbon dioxide acceptor ribulose 1,5-diphosphate. The enzyme catalysing the cleavage of ribulose 1,5-diphosphate, carboxydismutase[2], has been shown to be present in high activity only in micro-organisms grown on carbon dioxide, or on formate[5,6], or pentoses[7], and to be largely absent from such organisms grown on other carbon substrates[4-6]. Similarly, ribulose phosphate kinase has been shown[8] to be adaptively formed when *Pseudomonas* grown on oxalate continues to grow on formate, and to be "diluted out" when formate-grown cells continue to grow on oxalate. These considerations render it likely that the demonstration of the initial formation of 3-phosphoglyceric acid from carbon dioxide by *M. denitrificans* growing on carbon dioxide as sole carbon source (Fig. 1), and of the presence, in extracts of cells thus grown, of carboxydismutase, pentose phosphate isomerase and ribulose phosphate kinase (Table II), indicate the operation of the CALVIN cycle[3] in *M. denitrificans* during autotrophic growth.

Growth on acetate

Extracts of *M. denitrificans* grown on acetate did not contain significant quantities of carboxydismutase and of one or both of the other enzymes of the CALVIN cycle[3] for which tests were made (Table II), which showed that under these growth conditions this cycle did not operate. A similar indication of an alteration with change of carbon source of metabolic routes was provided by comparison of the ability of cell extracts to catalyse the chief component reactions of the glyoxylate cycle[12,13]. Both extracts obtained from cells grown autotrophically and on acetate were able to effect the activation of [1-^{14}C]acetate to [1-^{14}C]acetyl coenzyme A, albeit to different extents, and catalysed the condensation of [1-^{14}C]acetyl coenzyme A thus formed with oxaloacetate, to yield citrate, and with glyoxylate, to form malate. Only extracts of cells grown on acetate, however, catalysed the overall conversion (reaction

(I)) of [1-^{14}C]acetate to [1-^{14}C]malate in the presence of isocitrate (Table III). These findings support the view that the growth on acetate of *M. denitrificans*, like that of *M. sodonensis*[29], *Pseudomonas*[12, 13], *Escherichia coli*[30] and *Chromatium*[31], involves the functioning of the glyoxylate cycle (for review, see [32]). Since, of the three key enzymes of this cycle (condensing enzyme[16], malate synthetase[17] and isocitratase[19]), the first two were found in all extracts of *M. denitrificans* examined, under all conditions of growth (Tables III and IV), whilst the latter was present in high activity only in extracts of cells grown on acetate as sole carbon source (Table III), these results further strengthen the view[11] that the intracellular operation of the glyoxylate cycle is governed by the activity and formation of isocitratase.

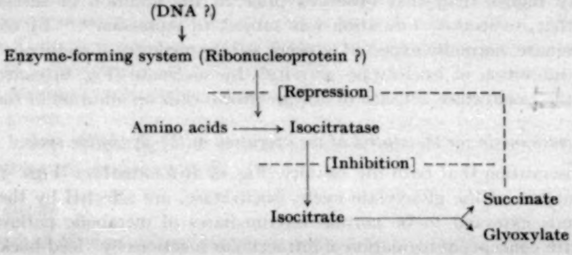
The influence of growth substrates on isocitratase formation

The finding (Table IV) that even cells grown on acetate did not contain significant amounts of isocitratase if small quantities of alternative carbon sources (in this case, yeast extract) were provided in the growth medium, indicated that the nature of the carbon source greatly influenced the operation of the glyoxylate cycle in this organism. This was also apparent when acetate-grown cells, which contained isocitratase in high activity, were placed in fresh growth media containing fumarate or malate (Fig. 3) or succinate (Fig. 4) as carbon sources. Whilst cells continuing to grow on acetate continued to form the enzyme at constant and high specific activity, cells growing on fumarate or malate formed the enzyme at less than 20 % of the former rate (Fig. 3) and those growing on succinate apparently ceased altogether to form the enzyme (Figs. 4 and 5). Addition of limited quantities of succinate to media containing acetate as chief carbon source, either initially (Fig. 4) or at intervals during the growth of the organisms (Fig. 5), resulted in a temporary cessation of isocitratase formation. After utilisation of the added succinate, isocitratase synthesis was resumed, at a rate considerably higher than that observed prior to the addition of succinate. This suggested that isocitratase formation was subject to repression[33–45] by compounds, such as succinate, normally expected to occur as intermediates of metabolic reactions. The direct inhibition of isocitratase activity[21] by succinate (Fig. 6) contributed to the fall in the isocitratase activity of acetate-grown cells on addition of succinate.

A possible mechanism for the control of the operation of the glyoxylate cycle

The observation that both the activity (Fig. 6) and formation (Figs. 3, 4 and 5) of a key enzyme of the glyoxylate cycle, isocitratase, are affected by the presence of compounds expected to be normal intermediates of metabolic pathways, is in harmony with concepts of regulation of intracellular reactions by "feed-back" control. Thus, for example, the work of VOGEL[33, 34], YATES AND PARDEE[35, 36], UMBARGER AND BROWN[37, 38], MAGASANIK and colleagues[39, 40] and GORINI AND MAAS[41–43] suggests that the intracellular levels of products produced by sequences of enzymic reactions control the rates at which early steps of such sequences operate (for reviews, see [44–46]) and that these controls are exerted both by enzymic inhibition and enzymic repression. This concept can also suggest a means of control of the intracellular operation of the glyoxylate cycle.

The repression of isocitratase formation by compounds other than acetate could be accomplished in a number of ways, including (a) repression of the formation of the enzyme-forming system, (b) repression of the formation of enzyme by the enzyme forming system, or (c) formation of an inactive or "masked" enzyme. Addition of chloramphenicol to *Pseudomonas* adapting to growth on acetate[11] arrested both the growth of the organism and the formation of isocitratase, which indicated that the elaboration of the enzyme during adaptation from succinate to acetate was not a consequence of the "unmasking" of an inhibited enzyme or an inactive precursor, but was associated with the *de novo* synthesis of protein, which therefore argued against postulate (c). Repression of the formation of the enzyme-forming system, as suggested under (a) above, would result in a "diluting out" of isocitratase when acetate-grown cells continue to grow on carbon sources other than acetate, as noted in Figs. 3, 4 and 5, but resumption of the formation of the enzyme-forming system, after relief of the repression, would not cause the immediate preferential synthesis of isocitratase observed. The findings that, after exhaustion of the succinate in media containing both acetate and succinate, isocitratase formation is more rapid than the formation of other cellular proteins[10] (Figs. 4 and 5), and that micro-organisms grown on carbon sources other than acetate still contain small but significant quantities of isocitratase[9–11, 19–22], indicate that cells capable of forming this enzyme are capable of so doing under all conditions, but realize this capability only when acetate or a precursor of acetate serve as carbon sources. This supports the postulate (b) that carbon sources other than acetate act by repressing the formation of isocitratase by the enzyme-forming system, but do not appreciably hinder the formation of the enzyme-forming system. Although no evidence is available that succinate itself acts as the regulator of intracellular isocitratase function, the results here presented support the hypothesis that isocitratase function, and hence the operation[10, 11] of the glyoxylate cycle[12, 13], can be controlled by feed-back mechanisms, in which the intracellular levels of succinate (or some materials derived from succinate) govern both the activity (Fig. 6) and formation (Figs. 3, 4 and 5) of isocitratase:



This hypothesis also agrees with the postulated biological role of the glyoxylate cycle. It has been concluded[32] that the pathway whereby acetate yields metabolic energy is the tricarboxylic acid cycle, but that the provision of carbon skeletons from acetate for the biosynthesis of cell constituents involves the functioning of the glyoxylate cycle (*cf.* [47]). Change from a milieu in which C_4-compounds are present to one in which acetate is the sole carbon source would thus be expected to result in an initial depletion of intracellular "pools" of intermediates and a consequent lowering of the intracellular levels of *e.g.* succinate, which in its turn would elicit the formation of isocitratase. Over-production of succinate, or external provision of C_4-compounds, would in consequence repress formation of the enzyme. This feedback system, coupled with the more immediately responsive control of inhibition of enzymic activity (Fig. 6), could serve as a factor in the maintenance of the balance of energy-yielding and energy-requiring reactions in cells growing under widely-differing conditions of nutritional environment.

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MICROSPECTROPHOTOMETRIC MEASUREMENTS OF THE CYTOCHROMES IN A MITOCHONDRIAL AGGREGATE *IN VIVO*

ROBERT PERRY*, BO THORELL**, LENNART ÅKERMAN** AND BRITTON CHANCE
Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. (U.S.A.),
Department of Pathology, Karolinska Institutet, Stockholm (Sweden)

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SUMMARY

Recent improvements in microspectrophotometry have allowed a quantitative *in vivo* assay of the respiratory enzymes in a single mitochondrial aggregate, the "nebenkern", of the grasshopper spermatid with a detectability equivalent to 6,000 molecules of cytochrome *b*. It has been possible to demonstrate Soret spectra of both oxidized and reduced cytochromes, depending on whether the cell is in an aerobic or an anaerobic state. The total concentration of cytochromes a_3, a, b, c, and c_1 in the "nebenkern" is calculated to be about $7 \cdot 10^4$ molecules/μ^3 and is at least 50 times that in any other comparable part of the cell.

INTRODUCTION

Microspectrophotometry of single living cells has been used extensively for the evaluation of nucleic acids, proteins, and other compounds existing in substrate

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Abbreviations used are: PNH (reduced pyridine nucleotide), IAA (iodoacetic acid).
* Present address: Laboratory of Animal Morphology, Free University of Brussels, Belgium.
** Permanent address: Department of Pathology, Karolinska Institutet, Stockholm, Sweden.

concentrations[1-3]. However, measurements on a single cell of the light absorption of compounds which are in enzyme concentrations have heretofore had only limited application[3] because the instruments used did not have sufficient sensitivity or stability for accurately recording the absorbancies of 0.1 to 1.0 % which are encountered. On the other hand, instruments capable of measuring these small absorbancies to a high degree of accuracy in suspensions of whole cells and cell fractions have been developed[4]. With such techniques a detectability of 10^{-11} moles of respiratory pigment in a 1-cm cuvette has been realized[5].

The obvious advantages of microspectrophotometry in localizing the measurements to specific parts of cells and in making it possible to select the cells on the basis of morphological or temporal criteria made it desirable to extend such highly sensitive measurements to the micro scale. The combination of sensitive recording circuits and the microscopic technique has now allowed the quantitative study of respiratory enzyme distributions in single living cells[6,7].

Whether or not the measurements can be localized to distinct structures within the cell such as mitochondria depends on the size and distribution of these structures. In some cases, favorable conditions can be attained by high speed centrifugation[8], although the resulting damage to the cell curtails the use of this technique for many types of problems. In other instances, there is a natural tendency for the mitochondria to concentrate themselves into a highly compact volume. Such an event occurs during spermatogenesis in certain insects when the "nebenkern" is formed.

In the grasshopper, the morphogenesis of the "nebenkern" has been elucidated by classical cytological methods, and more recently has been studied in detail by electron[9,10] and interference[11] microscopy. It appears to be formed in the spermatid by a coalescence of smaller mitochondria or mitochondrial filaments and later gives rise to the mitochondrial sheath of the sperm tail. Its solid concentration is not very different from that of the filaments, which suggests that there has been no gross modification of chemical organization during its formation[11].

This paper reports some spectrophotometric measurements made on the "nebenkern" in which a clearly defined Soret spectrum of the cytochromes is demonstrated.

METHODS AND MATERIALS

Apparatus

Since a detailed account of the instrument appears elsewhere[12], only a brief description will be given here.

A 6 V, 18 A, tungsten ribbon filament operated at 30 % over-voltage illuminates the entrance slit of a grating monochromator (Bausch & Lomb, 250-mm focus) which in turn illuminates the condenser of a microscope (Bausch & Lomb) by means of a quartz-fluoride lens arranged for Kohler illumination. Both the condenser and the objective are 1.6-mm Grey-Polaroid reflecting, water immersion components (N.A. 1.0). The effective slit width corresponds to a spectral interval of 3 mμ. A 3.5 X quartz ocular projects the beam alternately on each of two parallel non-coplanar mirrors vibrating at 60 cyc./sec. A suitable aperture is placed at the image plane and a 1P-28 photomultiplier is placed immediately beyond the aperture. The mirrors and aperture are so arranged that the light originating from two spots in the object plane, 1.5 μ in diameter and 12 μ apart, are put alternately on the same area of the photomultiplier. One of these beams is designated the measuring beam, the other the reference beam. The parts of the cell corresponding to the two spots are determined by means of an auxiliary telescope which can be interchanged with the photomultiplier.

The output of the photomultiplier contains coded information in the following format: dark, reference, dark, measure, each one of these corresponding to a certain potential output from the photomultiplier. It is the function of the electronic circuit to provide high amplification for the difference between the dark and the reference pulses in order to maintain this difference at exactly 1 V, in spite of variations of the emissivity of the light source and the sensitivity of the photocell. The electronic circuit also measures with a high degree of accuracy the difference between the amplitudes of the reference and measure pulse. The recorder is actuated by this difference. The specific advantages of the electronic circuits employed are that they provide a satisfactory method for the amplification of these small differences without amplifier overload due to the magnitude of the pulses themselves or to the large amount of noise which is inevitably carried along with the signal information. The amplification factor in the measurement of the dark-reference signal is $\sim$10-fold. That in the reference-measure circuit is sufficient that 10^{-4} in O.D. is readily detectable on the recorder (see Fig. 2). Considerable advantage is obtained from controlling the dynode voltage of the photocell from the difference between the reference and the dark signal since this is independent of the dark current of the photomultiplier and grid current of the amplifier tube.

In order to achieve rapid equilibration of the instrument and to facilitate the recording of spectra at these high sensitivities, auxiliary RC networks giving time constants (10–90 %) of either 5 or 10 sec were inserted across the output. The effect of these time constants on the cytochrome spectra was determined by recording the spectrum of the hemoglobin in an erythrocyte[13], which could be done at a much lower sensitivity without the auxiliary RC network (Fig. 6), and then inserting the particular RC and noting the displacement of the principal peak. The shifts were 5 and 7 mμ for the two above mentioned networks, and hence the wave lengths of all equivalent cytochrome Soret peaks were corrected by these amounts. No attempt was made to correct the peak height which, judging from the hemoglobin curves, was lowered by not more than 5 %.

Preparations

The biological preparations were made from mature grasshoppers according to the method described by BELAR[14]. A typical preparation consisted of the contents of one or two follicles suspended in about 3 μl of BELAR's buffer. The cells were confined between a normal slide and coverslip and the preparation sealed with paraffin. Both the "nebenkerne" which appear as single bodies (in early stage spermatids) and those which are double (in later stages) were measured.

Preparations made with normal BELAR's buffer, which contains glucose, go anaerobic in a few minutes, whereas if glucose is omitted they remain aerobic for about 1.5 h. The aerobic or anaerobic state of the preparation can be checked by mixing in a few red blood cells and then taking the hemoglobin spectrum of one of them[13].

For a comparison with the spectra of the individual "nebenkern", a bulk preparation of whole follicles was made from the testes of 10 grasshoppers. Using a double-beam spectrophotometer[4], it was then possible to obtain an anaerobic-minus-aerobic difference spectrum when nitrogen and oxygen were alternately passed over the follicular suspension.

RESULTS

Fig. 1 illustrates the absorption bands that might be expected from the respiratory pigments of the sperm and sperm precursor cells on the basis of studies of a whole follicle suspension. Follicles from 10 grasshoppers were placed in a moist chamber attachment for the double-beam spectrophotometer and the absorbancy changes occurring in the transition from aerobiosis to anaerobiosis were recorded point by point for a series of wave lengths from 340 to 500 mμ. It is seen that there are absorption bands due to three types of cytochromes (c, b, and a_3), flavoprotein, and reduced pyridine nucleotide.

The curves "A" of Figs. 2 and 3 are spectra obtained from a single "nebenkern" (NK) in two different anaerobic preparations; in both cases the reference area was free space (FS) outside the cell. Curve "B" of Fig. 2 was obtained when the cell was moved slightly so that the cytoplasm (CY) next to the "nebenkern" was in the

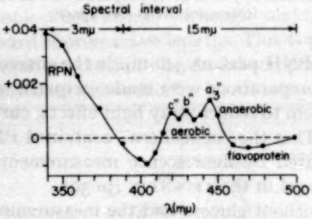

Fig. 1. Reduced minus oxidized difference spectrum of a suspension of whole follicles (904b).

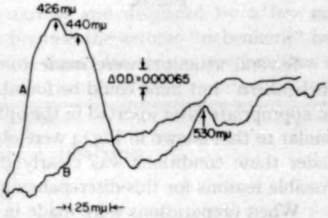

Fig. 2. Absorbance curves of a single anaerobic spermatid. A. Measuring area: "nebenkern", reference area: free space; B. Measuring area: cytoplasm, reference area: free space.

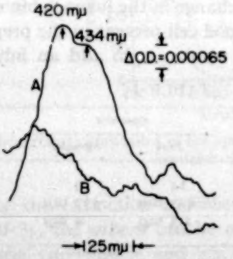

Fig. 3. Absorbance curves of a single anaerobic spermatid. A. Measuring area: "nebenkern", reference area: free space; B. Measuring area: nucleus, reference area: free space.

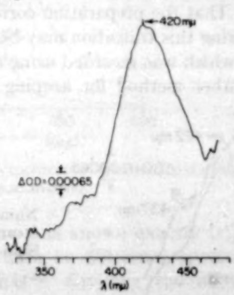

Fig. 4. Absorbance curve of anaerobic "nebenkern" extended to near-u.v. region. Reference area: cytoplasm. Time constant correction not applied to wave length scale.

measuring area while free space remained in the reference area. Curve "B" of Fig. 3 represents a similar case where the nucleus (NU) was moved into the measuring area. In the NK *vs* FS curves (A) of Figs. 2 and 3 there is a distinct peak at 420–426 mμ and a shoulder at 434–440 mμ, whereas the NU or CY *vs* FS curves (B) show no evidence of a peak. Clearly reproducible peaks in the region beyond 500 mμ have not yet been obtainable although the peak at 530 mμ seen in curve "A" of Fig. 2 has been observed many times.

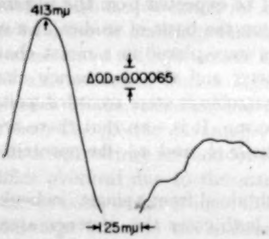

Fig. 5. Absorbance curve of "nebenkern" about 75 min after sealing the preparation. Reference area: nucleus. Preparation in buffer without glucose.

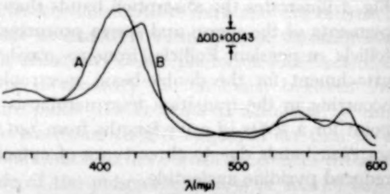

Fig. 6. Hemoglobin spectra of an erythrocyte present in a spermatid preparation which was made without glucose. A. Oxidized curve recorded less than 90 min after preparation was made. Reference area: free space. B. Reduced curve recorded 105 min after preparation was made Reference area: free space.

Several attempts were made to detect a PNH peak at 340 mμ in the anaerobic "nebenkern" but none could be found. When preparations were made on quartz and an appropriate filter inserted in the optical system to reduce stray light effects, curves similar to that shown in Fig. 4 were obtained. That the "nebenkern" contained PNH under these conditions was clearly demonstrated by fluorescence measurements[15]. Possible reasons for this discrepancy are discussed in the APPENDIX (p. 30).

When preparations were made in buffer without glucose and the measurements made within 75 min, the spectrum, illustrated in Fig. 5, was obtained. This curve has a single peak at approx. 413 mμ. If, however, measurements were taken about 90 min after the preparation was made, then the spectra resembled those of Figs. 2 and 3. That the preparation correspondingly went from an aerobic to an anaerobic state during this transition may be seen from the change in the hemoglobin spectrum (Fig. 6) which was recorded using a human red blood cell present in the preparation.

Another method for keeping a preparation aerobic is to add an inhibitor of

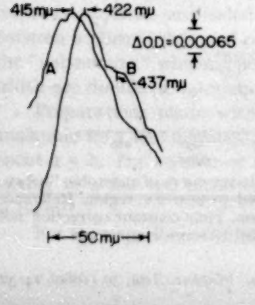

Fig. 7. Absorbance curves of "nebenkern" recorded about 30 min after preparation was made. A. Glucose-containing buffer plus 4 m*M* IAA. Reference area: nucleus. B. Glucose-containing buffer. Reference area: free space.

TABLE I

	Anaerobic		*Aerobic*
	Peak	*Shoulder*	
Number of records	14	9	8
Mean value	423 mμ	437 mμ	416 mμ
Standard deviation	2.5 mμ	4 mμ	1.5 mμ

respiration to the glucose-containing buffer. Preliminary experiments with the bulk suspension of follicles and an oxygen electrode showed that 4 m*M* IAA gave an appreciable inhibition of the respiration rate. Two preparations from the same testis were made in BELAR's buffer with glucose and 4 m*M* IAA was added to one. The curves, shown in Fig. 7, illustrate, within experimental variability, the same shift as that of Fig. 5.

A summary of the data on the "nebenkern" is given in Table I.

DISCUSSION

In identifying our spectra with those of the cytochromes, it is instructive to construct a theoretical composite of the curves for the isolated components. This is done in Fig. 8a where the γ-bands of the reduced cytochromes b^{16}, c^{17}, and a_3[18] are mixed in such a ratio that their sum gives a curve very similar to the experimentally observed anaerobic "nebenkern" curve. The peak at 420–426 mμ (Figs. 2 and 3) is contributed to mainly by cytochromes *b* and *c*, while the shoulder at 434–440 mμ comes principally from cytochrome a_3. The relative heights of the three constituent curves, which correspond to the relative products of extinction coefficient and molar ratio, are 1:0.9:1.6 for $c:b:a_3$. If the oxidized spectra of the isolated components are mixed in the same proportions (Fig. 8b) one obtains a curve similar to that of our aerobic "nebenkern" although slightly more narrow and displaced by a few mμ toward shorter wave lengths. This is probably because the aerobic "nebenkern" has a small proportion of reduced components as was found for aerobic preparations of isolated mitochondria[19].

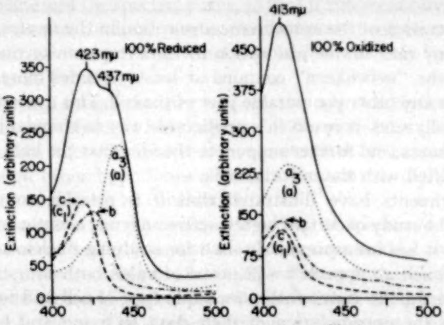

Fig. 8. Theoretical composites of the spectra of isolated cytochromes. (a) 100% reduced. (b) 100% oxidized. See text for details of mixing.

Only the extinction coefficient of cytochrome *c* is known exactly ($\varepsilon_{416}^{c''} = 129$ $\text{cm}^{-1}\,\text{m}M^{-1}$); the others can be estimated from data on difference spectra[19] to be $\varepsilon_{428}^{b''} \simeq 160$ $\text{cm}^{-1}\,\text{m}M^{-1}$ and $\varepsilon_{441}^{a_3''} \simeq 141$ $\text{cm}^{-1}\,\text{m}M^{-1}$. Dividing the above ratios by these values, we obtain 1:0.7:1.5 for the molar ratios of $c:b:a_3$. The cytochrome a_3 value includes some *a* (approx. 20 %[20]) and the *c* value some c_1 so that it is difficult to use the above to disprove a strict stoichiometry. It does seem, however, that there is an excess of terminal oxidase. Using an experimental value of 0.0048 O.D. units for the "nebenkern" at 423 mμ, we can estimate the concentration of cytochrome in the mitochondrion. Assuming 40 % contribution of cytochrome *b* to the peak and a 5 μ path length, we obtain 24 μM in cytochrome *b*. If the five principal cytochromes were present in equimolar amounts, the total concentration would be 120 μM or about $7 \cdot 10^4$ molecules/μ^3.

The relation between the μM concentration and the amount of cytochrome in μg/mg protein is

$$\mu M = c_s f_p \left(\frac{100}{M}\right) \frac{\mu\text{g cytochrome}}{\text{mg protein}}$$

where c_s is the per cent solid concentration, f_p is the per cent of the dry weight which is protein, and M is the molecular weight. From interference microscope data on the "nebenkern"[11], $c_s \simeq 25$. For a mitochondrion, f_p is about 65, and M for cytochrome *c* is 12,900. Hence, a 24 μM concentration of cytochrome *c* is equivalent to 2.0 μg/mg protein. This agrees very well with a value of 2.6 obtained from spectrophotometric measurements on a suspension of intact isolated mitochondria from rat liver[21], and is four times greater than a reported value for disrupted mitochondria isolated from rat liver[22]. Here is another indication that, in a chemical sense, the "nebenkern" may be considered a representative mitochondrion. From the partial specific volume and molecular weight of cytochrome *c*[23], one can calculate its molecular volume to be 10^{-8} μ^3. If we assume that the other cytochromes occupy approximately the same volume per molecule, we have $7 \cdot 10^{-4}$ μ^3/μ^3; that is, the cytochrome fraction of the respiratory chain occupies less than 0.1 % of the mitochondrial volume.

The demonstration of the cytochrome absorption in the *in vivo* "nebenkern" and the absence of any measurable absorption in the cytoplasm or nucleus leads to the conclusion that the "nebenkern" contains at least 50 times more cytochromes per unit volume than any other comparable part of the cell. This cytochrome is obviously active metabolically since it reacts in a predictable way to changes in oxygen tension. Thus our experiments lend further support to the idea that the bulk of the respiratory activity is associated with the mitochondria.

These experiments have illustrated that it is possible to use microspectrophotometry for the study of certain highly active enzymic components *in situ* in much the same way as it has been previously used for studying nucleic acids and proteins. It is hoped that such an approach will prove of value both to cytologists who wish to characterize metabolic states within a unique type of cell and to biochemists who wish to evaluate the extrapolation of their data to living and less heterogeneous systems.

APPENDIX

Errors

In estimating the errors related to the "nebenkern" structure and the microspectrograph optical system, the following factors are pertinent. (a) The Soret peaks are clearly defined with steep troughs on both sides. (b) The absorbancies being measured are very low ($\sim 5 \cdot 10^{-3}$). (c) The average refractive index quotients (calculated from the data of BARER AND JOSEPH[11]) are: $n_{NK}/n_{CY} = n_{CY}/n_{water}$ (434 mμ) = 1.01. (d) The diameter of the measured area is about 1/3 of the "nebenkern" diameter. (e) The "nebenkern" is a sphere and estimation of the measured volume to 10 % is possible even though the light is nonparallel.

Items (a) and (c) predict scattering, out-of-focus phase effects, and reflection losses make no appreciable contribution to the Soret region measurements. Furthermore, the very low real refractive index differences make it unlikely that anomalous dispersion is a significant source of error. Item (b) makes glare an unimportant factor and (d) minimizes effects owing to objective aberrations and gross inhomogeneities.

When estimating the uncertainty of the NK measurements it is helpful to use the NU, CY, or FS *vs.* FS curves as indicators of the wave length dependent nonspecific effects. These curves are usually monotonic functions which shift about $2 \cdot 10^{-3}$ O.D. units between 400 and 500 mμ. The direction of the shift is variable and although the illustrated curves for CY and NU go in opposite directions this is not a general rule. For these "base lines" to be perfectly flat it is required that the ratio of light emanating from the reference and measuring areas be exactly the same for all wave lengths observed. This is not rigorously attained in practice because first, the incident illuminations of the two areas do not have a relationship strictly independent of wave length, and second, the cytoplasm and nucleus are not optically homogeneous and occasionally a particle is present in the measuring or reference area which has scattering properties higher than those predicted by item (c). The first condition arises in spite of the fact that the monochromator grating is imaged on the object plane because there is an out-of-focus image of the slit, and hence the source, which exhibits some chromatic effect. This condition is the same for both the FS *vs.* FS base line and the spectral curve, so that it could conceivably be corrected for. However, the second condition varies from measurement to measurement, and hence causes an intrinsic uncertainty which can be lowered only by taking averages of several peaks, as in Table I.

In the 330–400-mμ range, the above effects are appreciably magnified and it is found that the base line shifts at least $1.5 \cdot 10^{-2}$ O.D. units for an FS–FS trace. It is likely that herein lies the principal reason for our lack of success in recording a PNH peak, although other hypotheses such as a localization of PNH on the mitochondrial periphery could also be considered.

A final source of error in the routine recording of spectra at these high sensitivities is the motion of material in the measuring area and/or the reference area. Only if the objects measured remain stationary within a fraction of a micron during the time necessary to record a spectrum (2 min for 100 mμ) can one obtain a meaningful and reproducible curve. The "nebenkern" is large enough so that Brownian motion is not a factor, as it might be in the case of smaller mitochondria under normal *in vivo* conditions.

In conclusion we make the following appraisal of our measurements. (a) The noise observed in the recording of a single spectrum with a 1.5-μ aperture is approx. 10^{-4} absorbancy units or about 2 % of the signal from a Soret peak. This is termed the detectability. If expressed in terms of cytochrome *b*, it represents about 6,000 molecules. (b) The average reproducibility encountered when one runs several curves of the same object is about $3 \cdot 10^{-4}$ absorbancy units or 6 %. This may be termed comparability. (c) From the considerations discussed in the preceding paragraphs it is estimated that the uncertainty of a given curve is ± 2 mμ for the wave length and ± 10 % for the peak height. (d) When making a calculation of the absolute concentration of cytochrome in a mitochondrion one has the above uncertainty compounded with the accuracy and applicability of the extinction coefficient and with the accuracy of the volume measurement (Item (e), p. 29).

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The authors are indebted to Dr. M. E. Gaulden for supplying them with grasshoppers and eggs of *Chortophaga viridisfaciata* and to Drs. E. H. Slifer and T. N. Tahmisian for eggs of *Melanopus differentialis*.

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SUR LA DÉTERMINATION DES SITES ACTIFS DE CERTAINES ENZYMES AU MOYEN D'UN NOUVEAU RÉACTIF SPÉCIFIQUE, LE SEL DE ROUSSIN. I

A. DOBRY-DUCLAUX
(avec la collaboration technique de Mlle N. DAUMAS)
Institut de Biologie physico-chimique, Paris (France)

(Reçu le 11 juin, 1959)

SUMMARY

Determination of the active sites of some enzymes by means of a new specific reagent: Roussin's salt. I

The inhibitory action of a mineral complex (Roussin's salt, $[Fe_4S_3(NO)_7]K$) on the respiration and fermentation of yeast and on six enzyme systems is described. The complex is a powerful inhibitor, acting at concentrations of the order of 10^{-5} to 10^{-7} M. It forms reversible bonds with the enzyme. It was found to compete with the substrates sodium pyruvate (carboxylase and lactic dehydrogenase) and DPN (alcohol dehydrogenase), and also acts as a coupling inhibitor or incompetitive inhibitor for: hexokinase–ATP, alcohol dehydrogenase–alcohol, and aldolase–fructose diphosphate. It does not compete with cocarboxylase (carboxylase), glucose (hexokinase), DPNH (lactic dehydrogenase). It also inhibits ribonuclease. The order of magnitude of the dissociation constant K_i is 10^{-5} to 10^{-7}, dehydrogenases being most sensitive to the action of the salt, and aldolase and ribonuclease least sensitive to it. The salt reacts only with one chemical group (see the following paper), and thus it permits us to ascertain the chemical nature of the prosthetic grouping of a certain number of enzymes.

INTRODUCTION

Dans le présent travail, je décrirai l'action inhibitrice d'un complexe minéral, dont l'action n'a jamais encore été essayée sur les systèmes enzymatiques: le sel de Roussin ou nitrososulfure de fer $[Fe_4S_3(NO)_7]$ K (abrégé: NSF). Ce sel agit sur une série d'enzymes, soit comme inhibiteur compétitif, soit comme inhibiteur de couplage. Dans le mémoire suivant, je montrerai qu'il n'agit qu'avec une seule fonction chimique. Cette substance permet donc d'identifier la nature chimique de l'un des centres actifs pour un certain nombre d'enzymes.

Propriétés générales du sel de Roussin

C'est un sel alcalin de l'acide $[Fe_4S_3(NO)_7]$ H. Ses propriétés sont inattendues et très différentes de celles d'autres complexes de fer, tels, par exemple, que les nitroprussiates. Par certains côtés, il se rapproche des complexes minéraux: l'acide est un acide fort, les solutions sont neutres et normalement ionisées. Mais contrairement à la plupart de ces complexes, il est très soluble dans les solvants organiques, même non ionisants. L'éther l'extrait de sa solution aqueuse. L'acide libre est insoluble dans l'eau, mais soluble dans le sulfure de carbone qui ne dissout presque aucun composé minéral.

Il a une coloration noire très foncée, si intense qu'elle est encore visible à l'oeil nu à la dilution de $2 \cdot 10^{-8}$ *M* sur une épaisseur de 1 cm. L'absorption est continue dans le spectre visible, avec un léger minimum à 700 mμ et une augmentation notable à partir de 480 mμ vers le violet. A 270 mμ, son coefficient d'extinction molaire est 12 fois plus grand qu'à 600 mμ ($\varepsilon_M^{0,270} = 2.32 \cdot 10^4$ exprimé en moles/l).

Notons encore qu'il agit aussi comme un désensibilisateur des plaques photographiques[1].

Action du sel de Roussin sur les organismes vivants

Le NSF a une action biologique remarquable. J'ai décrit dans un mémoire précédent son action bactéricide et bactériostatique[2]. Sa toxicité est très forte: il agit à des concentrations de l'ordre de 10^{-5} à 10^{-6} *M*. Il supprime la division cellulaire, empêche la croissance des racines, tue les protozoaires, les champignons etc. Depuis, A. CANDELI a repris cette étude[3] et constaté que le NSF agit aussi bien sur les bactéries gram-positives que sur les bactéries gram-négatives, en aérobiose et en anaérobiose. Il n'a pas pu élucider le mécanisme de son action et a seulement constaté qu'il est indépendant du potentiel d'oxydo-réduction.

Avant d'entrer dans le détail des expériences, il faut répondre à une objection possible. Les solutions diluées de NSF ne sont pas stables, surtout à la lumière. Les produits de décomposition sont les nitrates (non les nitrites), l'oxyde de fer et le soufre libre. On pourrait supposer que l'inhibition est attribuable à ces produits. Mais elle diminue à mesure que le complexe se décompose, jusqu'à devenir presque nulle. De plus, nous verrons que dans quelques cas l'inhibition est réversible, l'activité enzymatique reprenant sa valeur quand le NSF est extrait par l'éther, qui n'enlève pas les produits de décomposition éventuelle. C'est bien donc le NSF non altéré qui agit.

Observation au microscope des microorganismes en présence de NSF

Le NSF a le grand avantage sur beaucoup d'autres substances biologiquement actives d'être coloré, ce qui le rapproche, du point de vue des possibilités de travail, des atomes marqués. Une cellule de levure qui en a absorbé $1 \cdot 10^{-5}$ μg est colorée au microscope et, s'il se produisait à son intérieur une absorption sélective, elle serait immédiatement visible.

La première constatation qu'on peut faire, est que toutes les cellules, animales ou végétales, sont parfaitement perméables au NSF. Elles l'absorbent avec un très grand coefficient de partage en faveur de la cellule. Par example, la concentration à l'intérieur de la cellule est 700 à 1000 fois plus grande que dans le milieu environnant (les concentrations initiales de NSF variant de $7 \cdot 10^{-5}$ à $4 \cdot 10^{-4}$ *M* pour une suspension de levure à 0.066 mg de poids sec/ml).

Il ne semble pas y avoir de fixation préférentielle sur une partie de la cellule.

D'autre apart, le NSF agissant à doses très faible, son action par simple dénaturation des protéines semble exclue à priori. Les expériences suivantes confirment qu'on ne peut admettre l'action par dénaturation.

RÉSULTATS

Action sur la respiration et sur la fermentation alcoolique[*]

Ces deux phénomènes étant des processus enzymatiques primordiaux, on pouvait, grâce à eux, faire une étude préliminaire de l'action du NSF sur des systèmes enzymatiques indispensables à la vie. Les essais faits par la méthode classique, dans les manomètres de Warburg, ont montré que, parallèlement à la suppression de la division cellulaire, il y a un arrêt de la respiration et de la fermentation. La Fig. 1 montre la variation dans le temps de l'absorption d'oxygène en fonction de la quantité de NSF. La fermentation, mesurée par le dégagement de CO_2, donne des courbes analogues. Pour la même concentration de levure (30,000 à 32,000 cellules par mm^3), le taux d'inhibition est à peu près le même pour la respiration et pour la fermentation, soit 50 % pour une concentration de NSF de $6 \cdot 10^{-5}\,M$ et 90–95 % pour $1 \cdot 10^{-4}\,M$. Dans le mémoire antérieur[2], j'avais indiqué qu'il suffisait de $1 \cdot 10^{-6}\,M$ pour arrêter la croissance, soit 60 fois moins. Mais les expériences ne sont pas comparables, la concentration de la levure étant 50 à 60 fois plus faible dans les premières expériences (450 à 550 cellules par mm^3). La fixation du NSF étant pratiquement totale, on arrive à la même concentration de NSF à l'intérieur des cellules.

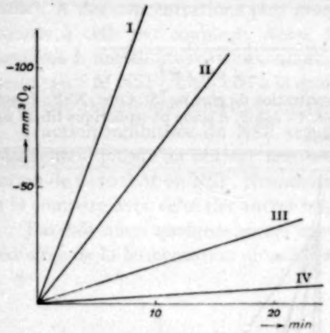

Fig. 1. Respiration de la levure de boulangerie. Conc. NSF: I, sans NSF; II, $6.5 \cdot 10^{-5}\,M$; III, $8 \cdot 10^{-5}\,M$; IV, $1 \cdot 10^{-4}\,M$.

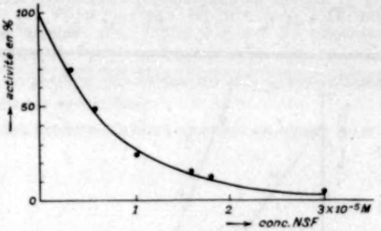

Fig. 2. Activité de l'hexokinase en présence du NSF, rapportée à l'activité de l'enzyme non inhibée.

Action sur les systèmes enzymatiques

Hexokinase

Les premiers essais ont montré que l'hexokinase est inhibée par des doses très faibles de NSF. La Fig. 2 montre la variation de l'activité (c'est à dire des vitesses initiales) en fonction de la concentration du NSF dans le mélange réactionnel.

Comme l'enzyme catalyse une réaction entre deux substrats, il fallait d'abord savoir quelle est la nature de l'inhibition par rapport à chacun de ces substrats. Cette détermination est basée sur l'étude de la cinétique de la réaction. J'ai choisi la représentation graphique des résultats selon Eadie[4], en étudiant la cinétique de la réaction en fonction de la concentration d'un des substrats. La Fig. 3 est le diagramme d'Eadie en fonction de la concentration en glucose. On constate que les vitesses initiales sont pratiquement indépendantes de la concentration en glucose, en présence et en absence de l'inhibiteur. Celui-ci est donc non compétitif vis à vis de glucose. La Fig. 4 se rapporte à la variation de la vitesse de la réaction en fonction de la concentration en ATP. Dans ce cas, le NSF agit comme inhibiteur de couplage.

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[*] Commencée en 1942 en collaboration avec Mme F. Boyer-Kawenoki.

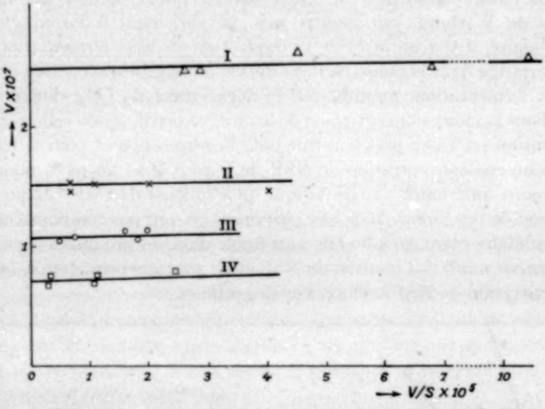

Fig. 3. Activité de l'hexokinase en fonction de la concentration du glucose (S). Conc. NSF: I, sans NSF; II, $4.34 \cdot 10^{-6}$ M; III, $8.68 \cdot 10^{-6}$ M; IV, $1.3 \cdot 10$ M. V: équiv. d'acide phosphorique libéré par min; S, moles/l.

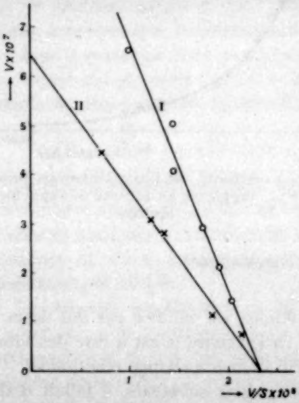

Fig. 4. Activité de l'héxokinase en fonction de la concentration de l'ATP (S). Conc. NSF: I, sans NSF; II, $6.6 \cdot 10^{-6}$ M.

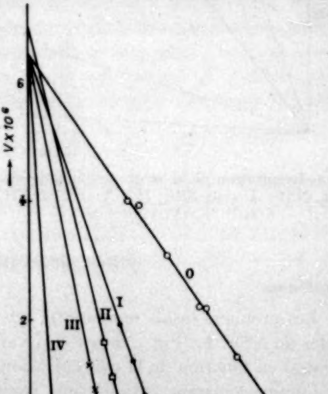

Fig. 5. Activité de la carboxylase en fonction de la concentration en pyruvate de Na (S). Conc. NSF: O, sans NSF; I, $3.8 \cdot 10^{-6}$ M; II, $6.1 \cdot 10^{-6}$ M; III, $7.6 \cdot 10^{-6}$ M; IV, $15.2 \cdot 10^{-6}$ M. V, moles de CO_2/min; S, moles/l.

Carboxylase

Le diagramme selon EADIE (Fig. 5) montre que le NSF est un inhibiteur compétitif vis à vis du pyruvate de Na. C'est de nouveau un inhibiteur puissant, puisqu'on obtient environ 50 % d'inhibition avec $8 \cdot 10^{-6}$ *M* de NSF.

Je n'ai pas déterminé avec précision la nature de l'inhibition par rapport à la co-carboxylase. Il semble qu'elle n'est pas compétitive, car l'addition de quantités de plus en plus grandes de co-carboxylase n'augmente pratiquement pas la vitesse de la réaction de l'enzyme partiellement inhibée, comme elle n'augmente pas, non plus, la vitesse de la réaction de l'enzyme non inhibée.

Aldolase

La Fig. 6 reproduit les résultats obtenus en fonction de la concentration du substrat. L'interprétation de ce diagramme est assez difficile, car les pentes des droites sont très faibles et leur point d'intersection est déplacé vers les grandes valeurs de V/S. Seul, un faible tronçon de chaque droite, se situant à des faibles valeurs de V/S est accessible à l'expérience. Mais des courbes analogues, obtenues avec d'autres enzymes, permettent d'élucider dans ce cas le mode d'action du NSF. Aux faibles pourcentages d'inhibition, il agit uniquement par couplage (ou comme inhibiteur incompétitif selon Eadie). A des concentrations plus grandes en NSF, une inhibition non compétitive s'ajoute à celle par couplage. Aussi, les droites V en fonction de V/S deviennent parallèles à une droite, correspondante à une inhibition maximale par couplage (ici avec $2 \cdot 10^{-5}$ *M* NSF), mais non à la droite de l'enzyme non inhibée. Il semble qu'on ne puisse pas obtenir une inhibition totale uniquement par le mécanisme de couplage.

L'action inhibitrice du NSF sur l'aldolase est plus faible que sur les enzymes précédentes, puisqu'on obtient une inhibition de 90 % seulement avec une concentration de $6 \cdot 10^{-5}$ *M* en NSF. Néanmoins, ce pouvoir inhibiteur est très grand, quand on le compare avec celui des autres inhibiteurs réversibles.

J'ai fait aussi quelques essais avec des enzymes autres que les enzymes intermédiaires de la fermentation alcoolique.

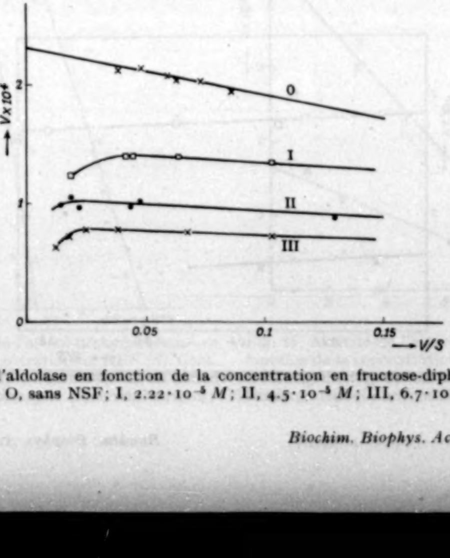

Fig. 6. Activité de l'aldolase en fonction de la concentration en fructose-diphosphate (*S*). Conc. NSF: O, sans NSF; I, $2.22 \cdot 10^{-5}$ *M*; II, $4.5 \cdot 10^{-5}$ *M*; III, $6.7 \cdot 10^{-5}$ *M*.

Ribonucléase

Sur la Fig. 7 est reproduite la diminution relative de l'activité de la ribonucléase en fonction de la concentration du NSF dans le mélange, l'activité en absence de l'inhibiteur étant posée égale à 100. Le NSF agit dans ce cas également comme inhibiteur, mais la ribonucléase nécessite une plus forte concentration en NSF que les autres enzymes. Je n'ai pas déterminé la nature de l'inhibition. Le NSF ne se fixe pas sur le substrat.

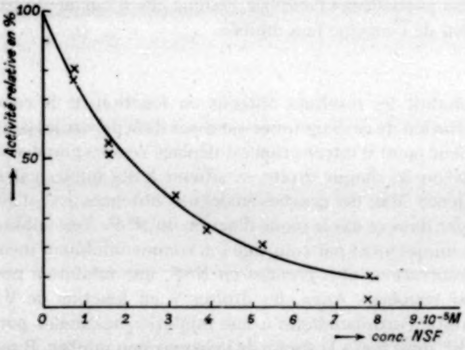

Fig. 7. Activité relative de la ribonucléase en fonction de la concentration molaire de NSF (activité de l'enzyme non inhibée = 100 %).

Lactico-deshydrogénase

La Fig. 8 reproduit le diagramme en fonction de la concentration en DPNH. On voit que le NSF est un inhibiteur non compétitif vis à vis de DPNH.

Par contre, l'inhibition est compétitive par rapport au pyruvate de Na, comme on le voit d'après la Fig. 9.

La lactico-déshydrogénase est plus sensible au NSF que toutes les enzymes précédentes.

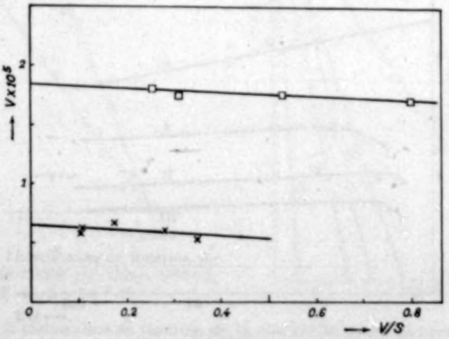

Fig. 8. Activité de la lactico-déshydrogénase en fonction de la concentration de DPNH (S). Conc. NSF: courbe supérieure, sans NSF; courbe inférieure, $1.64 \cdot 10^{-7}$ M.

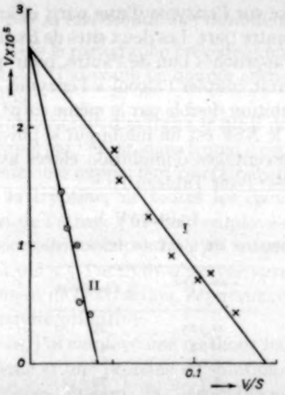

Fig. 9. Activité de la lactico-déshydrogénase en fonction de la concentration du pyruvate de Na (*S*). Conc. NSF: I, sans NSF; II, $1.64 \cdot 10^{-7}$ *M*.

Alcool-déshydrogénase

La Fig. 10 reproduit les résultats obtenus en fonction de la concentration en DPN. On constate que l'inhibition est compétitive par rapport au DPN. Les essais en fonction de la concentration en alcool n'ont été faits qu'avec une seule concentration en NSF et les résultats sont reproduits sur la Fig. 11. On peut en déduire que le NSF agit comme un inhibiteur de couplage (ou incompétitif) vis à vis du complexe enzyme-alcool. Le NSF produit donc une inhibition par double action: il se substitue au DPN

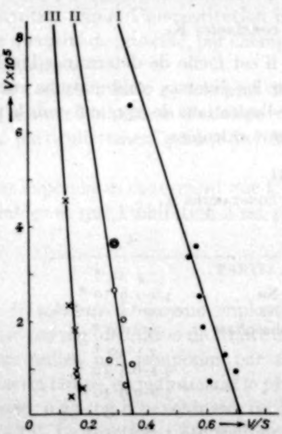

Fig. 10. Activité de l'alcool-déshydrogénase en fonction de la concentration de DPN (*S*). Conc. NSF: I, sans NSF; II, $1 \cdot 10^{-6}$ *M*; III, $1.64 \cdot 10^{-6}$ *M*; *V*, moles/min; *S*, moles/l.

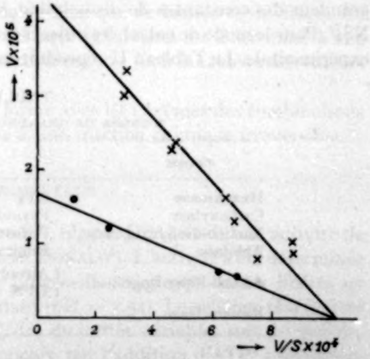

Fig. 11. Activité de l'alcool-déshydrogénase en fonction de la concentration en alcool (*S*). Conc. NSF: courbe supérieure, sans NSF; courbe inférieure, $1 \cdot 10^{-7}$ *M*; *V*, moles/min; *S*, moles/l.

à l'endroit où celui-ci se fixe sur l'enzyme d'une part, et empêche la dissociation du complexe alcool–enzyme d'autre part. Les deux sites de fixation, celui du DPN et celui d'alcool, doivent être très rapprochés l'un de l'autre, pour que le NSF, fixé sur le site de DPN, puisse de cet endroit coupler l'alcool à l'enzyme. A ma connaissance, c'est l'unique exemple d'une inhibition double par le même corps. C'est peut être, par suite de cette double action que le NSF est un inhibiteur si puissant de l'alcool deshydrogénase: on obtient des pourcentages d'inhibition élevés avec des concentrations de NSF particulièrement faibles: (voir Tableau I).

TABLEAU I

INHIBITION DE L'ALCOOL-DÉSHYDROGÉNASE

Conc. NSF *10^{-7} M*	*% d'inhibition*
0.325	29
0.54	41
1.08	61
2.7	72
5.4	78
10.8	85
21.6	88
54	97

Pepsine et trypsine

Les essais avec ces enzymes se sont heurtés à une grande difficulté: l'inhibiteur a autant d'affinité pour le substrat (la lactoglobuline et la sérumalbumine) que pour les enzymes. Comme la concentration en substrat est généralement 200 à 500 plus grande que celle d'enzyme, la plus grande quantité de NSF est fixée en réalité par le substrat.

Dans les conditions employées, il n'y avait aucune inhibition jusqu'à $1 \cdot 10^{-4}$ *M* en NSF.

L'ordre de grandeur des constantes K_i

A partir de tous les diagrammes obtenus, il est facile de déterminer l'ordre de grandeur des constantes de dissociation K_i pour les diverses combinaisons enzyme–NSF. Pour le mode de calcul, les objections et les limitations de ce calcul, voir la partie expérimentale. Le Tableau II reproduit les valeurs calculées.

TABLEAU II

L'ORDRE DE GRANDEUR DES CONSTANTES K_i

Enzyme	*Substrat*	K_i
Hexokinase	ATP	$7.7 \cdot 10^{-6}$
Carboxylase	Pyruvate de Na	$3.6–1.8 \cdot 10^{-6}$
Lactico-déshydrogénase	Pyruvate de Na	$6 \cdot 10^{-8}–2 \cdot 10^{-7}$
Aldolase	Fructose–diphosphate	$1–2 \cdot 10^{-5}$
Alcool-déshydrogénase	Alcool	$7.1 \cdot 10^{-7}$
	DPN	$9.4–6.5 \cdot 10^{-7}$
Ribonucléase		$3.6–0.9 \cdot 10^{-5}$

Les valeurs de K_i seront utilisées surtout pour l'identification du groupement chimique de l'enzyme sur lequel le NSF se fixe. (Voir le mémoire suivant.) Il suffit alors d'en connaître seulement l'ordre de grandeur.

Sur la réversibilité de l'inhibition

Les résultats discutés dans le paragraphe précédent ont montré que les complexes NSF–enzyme sont dissociables. J'ai voulu en donner encore une preuve, en montrant que l'inhibition est réversible.

La méthode la plus simple serait d'extraire le NSF de la solution inhibée par un liquide organique, par exemple par l'éther, dans lequel il est plus soluble que dans l'eau. Malheureusement, peu d'enzymes supportent ce traitement sans perdre leur activité. Seules la ribonucléase et la trypsine, de toutes les enzymes essayées, résistent en solution aqueuse à l'action de l'éther. J'ai donc employé cette méthode uniquement pour la ribonucléase. La réversibilité dépend du pH auquel l'extraction est effectuée. Quand on extrait le NSF à pH 5.5, l'activité observée ensuite est nulle. Par contre, si le pH est égal à 7.8 au moment de l'extraction, l'épuisement du NSF est intégral et on recupère 76 à 80 % de l'activité primitive.

Pour les autres enzymes, j'ai employé une méthode indirecte, basée sur le partage de l'inhibiteur entre l'enzyme et une protéine en solution.

Les protéines employées étaient la sérumalbumine et la lactoglobuline cristallisées*, les enzymes: l'hexokinase, l'aldolase et la lactico-déshydrogénase. Je me suis assurée que les protéines employées n'activent pas à elles seules ces enzymes dans les conditions employées.

Beaucoup de protéines absorbent le NSF. Aussi, si l'on ajoute à une solution d'une enzyme, préalablement inhibée, de la protéine, le NSF libre se fixe sur celle-ci et l'équilibre entre l'enzyme et le NSF est déplacé. Il devrait en résulter une diminution du degré d'inhibition. Effectivement, dans tous les cas essayés, l'inhibition diminue après l'addition de la protéine et d'autant plus que la quantité ajoutée est plus grande. Ainsi, pour l'aldolase et pour l'hexokinase, on obtient environ 40 % de diminution d'inhibition par l'addition du même poids de la protéine que de l'enzyme et 70 % par une quantité triple. Une restitution intégrale de l'activité ne s'obtient qu'avec une grande quantité de protéine, par exemple avec 100 fois plus en poids de sérumalbumine que d'aldolase.

Par contre, il faut 45 fois plus de lactoglobuline que de lactico-déshydrogénase pour diminuer l'inhibition d'environ moitié (de 74 à 43 %). Ceci est à attribuer à une affinité particulièrement grande du NSF pour cette enzyme (voir le tableau des valeurs K_i).

Ces expériences confirment que le NSF forme avec les enzymes des combinaisons dissociables et que l'inhibition n'est pas due à une réaction chimique irréversible.

PARTIE EXPÉRIMENTALE

I. Hexokinase. L'enzyme employée ("crude", Sigma, USA) avait une activité de 75 unités par mg (définition de l'unité d'après McDONALD[5]). L'activité a été déterminée dans un milieu non tamponné par titrage progressif des groups acides libérés en fonction du temps, en maintenant le pH constant (pH = 7.54). Le mélange réactionnel contenait: 0.25 mg d'héxokinase, de $MgCl_2$, des quantités variables soit de glucose, soit d'ATP. La réaction a été toujours déclanchée par l'addition d'ATP; l'inhibiteur a été ajouté après avoir protégé l'enzyme par le glucose. Volume initial 5.0 ml. Le pH des réactifs a été ajusté au préalable de telle manière qu'on obtienne après le mélange immédiatement le pH désiré.

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* Mises à ma disposition par Mlle J. YON, à laquelle j'adresse mes remerciements.

II. Carboxylase. L'enzyme a été préparée à partir de la levure de bière selon les indication de TH. P. SINGER[6]. La fraction employée avait un rapport d'activité égal à 0.42, c'est à dire qu'elle était presque aussi pure que la plus pure fraction de cet auteur (rapport d'activité égal à 0.39). Pour les essais manometriques, le mélange réactionnel contenait: 1 unité d'enzyme, 0.3 ml de tampon citrate 0.5 M de pH 6.0, des quantités variables de NSF, de $MgCl_2$ et 0.5 ml de pyruvate de Na 0.2 M ou 0.1 M ou 0.05 M, dans un volume total de 3.3 ml.

III. Aldolase, cristallisée du muscle (Boehringer, Allemagne). Son activité a été déterminée selon J. F. TAYLOR[7], par la méthode chimique, en dosant l'acide phosphorique libérable par saponification alcaline. La concentration de l'aldolase a été choisie de manière qu'au bout de 5 min d'action à 30°, il y avait au maximum 50 % de fructose-diphosphate transformé en triosephosphates, afin de pouvoir admettre que la quantité formée d'acide phosphorique labile est proportionelle à la vitesse initiale de la réaction. L'aldolase avait une activité de 2000 unités, définies selon TH. BÜCHER *et al.*[8]. Le mélange réactionnel contenait: 0.1 ml de solution d'aldolase dans KCN 0.05 M (correspondant à 12.1 μg d'aldolase), 0.02 à 0.30 ml de sel de Na de fructose-diphosphate 0.01 M dans glycocolle 0.1 M pH 9.0, des quantités variables de NSF, dans un volume total de 0.45 ml. Dans les expériences de réversibilité de l'inhibition par les protéines, le mélange contenait une quantité double d'enzyme, 0.1 ml NSF à 55.2 μg/ml, 0.1 ml de fructose-diphosphate, des quantités variables de protéine, le tout dans un volume de 0.8 ml. On déféquait avant le dosage du phosphore.

IV. Ribonucléase (Sigma, USA). Les mesures d'activité ont été faites d'après les indications de M.R. MCDONALD[9]. Le mélange réactionnel contenait 5 mg d'acide nucléique commercial, purifié d'après KUNITZ[10], 9.5 μg de ribonucléase et des quantités variables de NSF dans un volume de 2.5 ml. Temps de digestion 120 min à 30° et à pH 4.55.

V. Lactico-déshydrogénase (Boehringer, Allemagne) était très pure, elle avait 213 unités/mg (définies selon KORNBERG[11]). Pour les dosages d'activité, on a suivi les indications de cet auteur, sauf que la réaction a été déclanchée par l'addition de pyruvate de Na au lieu de l'être par l'enzyme. L'enzyme a été protégé par le DPNH avant l'addition de l'inhibiteur.

VI. Alcool-déshydrogénase de la levure (Boehringer) était pure: son activité spécifique (selon E. RACKER[12]) était 155,000 unités/mg. Les déterminations ont été faites avec 1 μg d'enzyme/ml, d'après les indications du même auteur. L'enzyme, protégée par le DPN, a été additionnée de NSF et la réaction a été déclanchée par addition de l'alcool et non par celle d'enzyme.

VII. Pepsine et trypsine cristallisées (Worthington Biochemical Corp.).

VIII. Expériences sur la réversibilité de l'inhibition (par l'extraction du NSF par l'éther). 2.0 ml d'une solution contenant 45 μg de ribonucléase, 0.5 ml de NSF à 0.22 mg/ml et 1 goutte de tampon acétate de pH = 5.5 sont agités 4 fois avec leur volume d'éther. Le NSF passe dans la couche éthérée. Celle-ci devient incolore après 3 extractions, mais la couche aqueuse reste légèrement teintée. Après avoir chassé l'éther de la couche aqueuse par le vide, on y ajoute de l'acide nucléique et on effectue la détermination de son activité dans les conditions usuelles. Pour l'extraction à pH 7.8, on emploie le tampon phosphate à la place du tampon acétate et on acidule après l'extraction. La ribonucléase, seule, est soumise au même traitement et on rapporte à son activité, l'activité recupérée après l'inhibition par le NSF.

IX. Mode de calcul des constantes de dissociation K_i. Pour pouvoir calculer les constantes K_i, il faut connaître, dans chaque cas, la concentration en inhibiteur *libre*. Cette condition a souvent été négligée. Dans la plupart des cas, les auteurs ont employé la concentration globale d'inhibiteur à la place de la concentration libre. Cette substitution ne conduit à une erreur négligeable que lorsque l'affinité de l'inhibiteur pour l'enzyme est faible, c'est à dire la constante K_i relativement grande. Sinon, on obtient des valeurs de K_i trop élevées.

Or nous avons constaté que le NSF a une très grande affinité pour les enzymes étudiées. Il aurait fallu connaître les concentrations en NSF libre. Malheureusement, ceci était pratiquement impossible: le NSF ne change pas de spectre d'absorption par fixation sur une protéine, comme le font certains colorants. L'ultrafiltration demande des quantités prohibitives d'enzymes pures et n'est pas suffisamment sûre avec des concentrations aussi faibles d'inhibiteur. Aussi, je me suis bornée au calcul de la *limite supérieure* des K_i, en prenant la concentration globale à la place de la concentration en inhibiteur libre. Les vraies valeurs de K_i sont donc encore plus petites que celles indiquées dans le Tableau II. Par suite de cette approximation, on ne peut pas obtenir des valeurs de K_i indépendantes des quantités employées de substrat ou d'inhibiteur. Aussi, j' indique sur le Tableau II les limites supérieures et inférieures des constantes de dissociation calculées à partir des données expérimentales.

DISCUSSION

La discussion est placée à la fin du mémoire suivant, lorsque la nature chimique du groupe actif, sur lequel le NSF se fixe, sera définie.

REMERCIEMENT

Je remercie bien sincèrement Mme Y. KHOUVINE qui a bien voulu se priver pour moi de divers appareils sans lesquels ce travail n'aurait pas pu être fait.

RÉSUMÉ

On décrit l'action inhibitrice d'un complexe minéral (sel de Roussin ou nitrososulfure de fer, $[Fe_4S_3(NO)_7]$ K) sur la respiration et la fermentation de la levure, ainsi que sur six systèmes enzymatiques. C'est un inhibiteur très puissant, agissant à des concentrations de l'ordre 10^{-5} à 10^{-7} *M*. Il se fixe réversiblement sur l'enzyme. Il est compétitif avec les substrats suivants: le pyruvate de Na (carboxylase et lactico-deshydrogénase), le DPN (alcool-deshydrogénase). Il agit comme inhibiteur de couplage (ou incompétitif) pour: l'hexokinase–ATP, l'alcool-deshydrogénase–alcool, et l'aldolase–fructosediphosphate. Il est non compétitif pour la co-carboxylase (carboxylase), le glucose (hexokinase), le DPNH (lactico-deshydrogénase). Il inhibe aussi la ribonucléase. Les ordres de grandeurs des constantes K_i sont de 10^{-5} à 10^{-7}, les deshydrogénases étant les plus sensibles à l'action de ce sel, l'aldolase et la ribonucléase les moins sensibles. Il ne réagit qu'avec une seule fonction chimique (voir mémoire suivant) et permet ainsi d'identifier la nature chimique du groupement prosthétique pour un certain nombre d'enzymes.

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SUR LA DÉTERMINATION DES SITES ACTIFS DE CERTAINES ENZYMES AU MOYEN D'UN NOUVEAU RÉACTIF SPÉCIFIQUE, LE SEL DE ROUSSIN. II

A. DOBRY-DUCLAUX
(avec la collaboration technique de Mlle N. Daumas)
Institut de Biologie physico-chimique, Paris (France)

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SUMMARY

Determination of the active sites of some enzymes by means of a new specific reagent: Roussin's salt. II

In the preceding paper[1] we have shown that Roussin's salt ($[Fe_4S_3(NO)_7]K$) is a powerful inhibitor of a series of enzymes. Frequently it competes with the substrate.

To determine the chemical character of the active site of the enzyme, the functional group which reacts reversibly with the inhibitor must be known. Of all known functional groups of proteins, only the NH_3^+ group and the NH group of imidazole can combine with Roussin's salt, forming a dissociable salt. The solubility, as well as the dissociation constant of the compounds obtained with the various amines diminish with the increase of the number of amino groups per molecule, and especially with their increasing proximity. This intimates the possibility of chelation.

To find an explanation for the low K_i values obtained for the enzymes studied, we must assume that the active site of the enzymes to which Roussin's salt becomes attached is made up of several amino or imidazole groups lying very close to one another. Presumably, they are the position of fixation of the DPN$^+$ and of pyruvic acid (in the case of the carboxylase, alcohol dehydrogenase and lactic dehydrogenase). The sites of attachment of alcohol (alcohol dehydrogenase), of ATP (hexokinase) and of fructose diphosphate (aldolase) appear to lie in the immediate vicinity of these amino groups, since the Roussin's salt, when attached to these groups acts as a coupling inhibitor for these substrates. In two enzymes: alcohol dehydrogenase and lactic dehydrogenase, the SH-group—the second active group—lies next to the basic groups.

INTRODUCTION

Dans le mémoire précédent[1], j'ai décrit l'action inhibitrice d'un complexe minéral, le sel de Roussin ou nitrososulfure de fer (NSF), sur une série d'enzymes. J'ai pensé que cette action permettrait de déterminer par une voie nouvelle la nature chimique du site actif de l'enzyme et par suite de confirmer ou d'étendre les résultats déjà acquis.

RÉSULTATS

Action du NSF sur la fonction thiol

Pour l'activité de la plupart des enzymes essayées, la fonction SH est indispensable Il s'agissait en premier lieu de savoir si c'est sur ce groupe que le NSF se fixe.

Je n'ai pu mettre en évidence aucune action du NSF sur les groupes SH ou S–S. Le coefficient de partage du NSF entre une solution aqueuse et une phase organique liquide ou solide (alcool butylique secondaire, soie naturelle) a été le même en présence de corps à fonction thiol ou thiol oxydé (cystéine, acide thiomalique, glutathion et forme oxydée de ces deux dernières substances). La réactivité du SH n'a pas été modifiée. Les groupes S–S ne sont pas réduites par le NSF.

L'hypothèse d'une action du NSF par l'intermédiaire de son potentiel d'oxydo-réduction n'est pas soutenable. En effet, son rH est égal à 16 d'après mes mesures et à 15.5 d'après celles de TREADWELL ET HUBER[2], valeurs très voisines du rH de la plupart des liquides biologiques. Cette hypothèse est d'ailleurs exclue par les résultats données dans le premier mémoire.

Action du NSF sur diverses autres fonctions chimiques

Le but du travail est de reconnaître les fonctions chimiques qui réagissent sur le NSF en donnant des combinaisons dissociables. Seules peuvent être prises en considération les combinaisons pour lesquelles la constante de dissociation est du même ordre de grandeur que les constantes K_i trouvées pour les complexes enzyme-NSF.

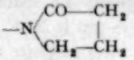
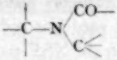
Pour juger les résultats, il faut aussi tenir compte de la grosseur de la molécule. Il est possible que dans une enzyme plusieurs groupes identiques se trouvent voisins les uns des autres et ce voisinage modifie leurs propriétés. En particulier, la constante de dissociation des complexes qu'ils forment avec le NSF peut n'être pas la même que pour un groupe isolé et nous verrons dans la suite de ce mémoire qu'elle semble en effet être beaucoup plus petite.

Aussi, pour déterminer l'ordre de grandeur de la constante de dissociation du complexe formé par une fonction avec le NSF, et en particulier les valeurs les plus faibles de cette constante, il est préférable d'opérer avec les hauts-polymères, porteurs de diverses fonctions en nombre comparable à celui qui existe dans les protéines. De cette manière, les distances mutuelles de ces groupes, qui sont un élément important, sont voisines de ce qu'elles sont dans les enzymes.

L'emploi de hauts-polymères simplifie en même temps le mode opératoire: leur nature macromoléculaire permet l'emploi de membranes sémiperméables grâce auxquelles il est facile de déterminer la concentration du NSF libre en équilibre avec la combinaison formée, et de calculer directement la constante de dissociation.

Les polymères employés et les résultats obtenus sont indiqués sur le Tableau I. Je n'ai pas trouvé de polymère constitué par les groupes phénoliques ou indolique correspondants aux restes de tyrosine ou de tryptophane. Mais les essais faits avec ces deux acides aminés ont montré qu'ils ne se combinent pas au NSF. La tryptamine se combine au NSF et précipite de sa solution aqueuse, mais les cristaux ne contiennent qu'un reste de NSF par molécule de tryptamine, correspondant à son groupe aminé salifié, et non à l'indole. Par contre l'imidazole se combine au NSF.

TABLEAU I

Monomère	*Polymère* $(\text{—CH—CH}_2\text{—})_n$ R *avec R:*	*Groupe susceptible de se combiner au NSF*	*Absorption par le polymère*	*K*
Acrylate de Na	—COONa	COO^-	—	
Acrylamide	—CONH_2	CONH_2	—	
Alcool vinylique	—OH	OH	±	$6 \cdot 10^{-3}$
Méthylolacrylamide	$\text{—CONHCH}_2\text{OH}$	CO—NH et OH	—	
Vinylpyrrolidone			++	$1.2 \cdot 10^{-3}$
Chlorhydrate de vinylamine	$\text{—NH}_3\cdot\text{Cl}$	NH_3^+	+++	$< 1 \cdot 10^{-6}$

Légende: — pas de combinaison;
± formation d'une combinaison très dissociée;
++ formation d'une combinaison dissociée et soluble;
+++ formation d'une combinaison insoluble;
K = constante de dissociation.

L'examen de ces résultats montre que:

(1) Ni le groupe carboxyle, ni le groupe amide ne se combinent au NSF.

(2) On pourrait penser que le groupe OH a une certaine affinité pour le NSF, puisque l'alcool polyvinylique commercial en absorbe. Mais cette absorption est très faible; en présence d'un excès de NSF un seul groupe monomère sur 400 en a fixé. Deux hypothèses sont possibles: ou bien le NSF se fixe réellement sur OH et en ce cas la constante de dissociation est égale à $6 \cdot 10^{-3}$, chiffre beaucoup plus grand que celui trouvé pour les enzymes; ou bien la fixation se fait non sur le OH, mais sur les groupes COOCH_3, dont il subsiste un certain nombre dans l'alcool polyvinylique commercial. Le NSF se fixe en effet sur l'acétate de cellulose qui a aussi des groupes esters. De toute manière, le groupe OH ne peut être retenu comme groupe actif des enzymes susceptibles d'être inhibées par le NSF.

(3) Le cas de la polyvinylpyrrolidone doit être examiné de près. Le groupement chimique de ce corps, qui fixe le NSF, est identique à celui d'une liaison peptidique, formée par des restes de sarcosine, $\text{CH}_3\text{NH–CH}_2\text{COOH}$. Ce polymère peut ainsi servir comme modèle de cette liaison dans les protéines. Comme la polyvinyl-pyrrolidone, l'anhydride de sarcosine, se combine au NSF, à raison de 1 molécule de NSF par molécule d'anhydride, en donnant un corps relativement peu soluble dans l'eau (1/2700). Mais c'est un produit d'addition et non un sel, résultant d'une double réaction

ionique, comme c'est le cas avec les amines (voir plus loin). Le NSF s'y comporte comme une multitude de substances organiques, qui forment des produits d'addition bien définis avec l'anhydride de sarcosine[3]. D'après les équilibres de dialyse, la constante de dissociation est $1.2 \cdot 10^{-3}$. Elle est trop grande pour qu'une liaison peptidique, formée par des restes de sarcosine, puisse être admise comme groupement actif des enzymes étudiées.

(4) Ni la liaison peptidique ordinaire –CO–NH– (étudiée avec la polyméthylol-acrylamide), ni l'anhydride de glycocolle (diacipipérazine) ne se combinent au NSF. Il est intéressant de noter que la substitution à l'azote d'un atome d'hydrogène par un groupe méthylé, modifie profondement l'affinité de la liaison peptidique pour le NSF.

(5) Tous les groupes aminés du chlorhydrate de polyvinylamine se combinent au NSF, en donnant un roussinate* complètement insoluble et du KCl. Il n'est pas possible dans ces conditions de déterminer la constante de dissociation du sel formé. Mais si la quantité ajoutée de NSF ne dépasse pas un dixième des groupes aminés, le polymère reste soluble et il est possible d'effectuer les mesures par dialyse sur une membrane de cellophane. L'eau d'équilibre, ainsi que l'ultrafiltrat sont absolument incolores sur une épaisseur de 20 cm, tandis que la solution du polymère est noire. Or on peut constater à l'oeil nu une coloration quand l'eau contient 0.2 μg NSF/ml. En prenant la moitié de cette valeur comme limite supérieure de la concentration du NSF dans l'ultrafiltrat, on obtient comme *limite supérieure* de la constante de dissociation la valeur $K = 2 \cdot 10^{-6}$, du même ordre de grandeur que les K_i des enzymes etudiées.

(6) Il ne reste donc que le groupe aminé ainsi que le groupe imidazole qui puissent retenir notre attention.

J'ai montré en 1941[4] que le NSF se combine avec beaucoup d'amines en proportions définies, en donnant des combinaisons du type salin, presque toujours peu solubles dans l'eau et facilement cristallisables. J'ai obtenu les roussinates de 41 bases aminées, aliphatiques, aromatiques, bases hétérocycliques azotées et d'acides aminés bibasiques.

L'existence de ces composés ne répond pas parfaitement à la question posée, qui est celle des constantes de dissociation réversible. En effet, la formation d'un composé insoluble, par fixation d'un réactif sur une fonction chimiqiue, est un phénomène différent de l'inactivation de cette fonction par formation d'un sel peu dissocié, car ce sel peut être très soluble. Mais dans le cas présent, les deux phénomènes peuvent être confondus, et en fait il le sont toujours en enzymologie, sans même que la question soit posée. Le point essentiel est que le groupe actif est neutralisé, d'une manière ou d'une autre.

Influence de la répartition des groupes aminés

Nous pouvons maintenant aller plus loin et essayer d'atteindre le nombre et la configuration des groupes aminés dans les centres actifs des enzymes inactivées par le NSF, en profitant de leur action mutuelle quand ils sont voisins.

Dans ce but, nous allons comparer les constantes de dissociation des roussinates des monoamines aliphatiques avec celle du roussinate de la polyvinylamine. Ces constantes sont: $3.2 \cdot 10^{-3}$ (éthylamine), $6 \cdot 10^{-3}$ (lysine) et $< 1 \cdot 10^{-6}$ (polyvinylamine).

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* Les sels de NSF (ou sel de Roussin) sont appelés les roussinates.

La constante du polymère est donc au moins 1000 fois plus petite que celle du monomère (éthylamine). Quelle est la raison de cette différence?

Le roussinate de polyvinylamine étant un polyélectrolyte, la discussion de ce problème ne peut être faite qu'en le rattachant aux propriétés générales des polyélectrolytes, telles qu'elles ont été établies en particulier par KERN, KATCHALSKY, HERMANS, BOURDAIS, DOBRY et autres auteurs[5–9]. Ces auteurs ont mesuré la dissociation et l'activité d'un grand nombre de polyélectrolytes synthétiques et l'ont comparé à celles des monomères correspondants. Ils ont trouvé que la dissociation d'un polyélectrolyte est toujours moindre que celle du monomère correspondant, mais généralement du même ordre de grandeur. BOURDAIS a trouvé ainsi pour des solution de 0.01 M (en unités de monomère):

	Degré de dissociation α
Acide propionique (monomère)	$\alpha = 0.036$
Acide polyacrylique	$= 0.02$
Propionate de Na (monomère)	$= 0.92$
Polyacrylate de Na	$= 0.40$

Mais cette différence diminue exponentiellement au fur et à mesure que les groupes ionisés (par exemple les groupes carboxyles) *s'éloignent les uns des autres le long de la chaîne polymérique*. Ceci s'obtient en formant des copolymères entre un acide et un non acide (acrylamide) ou en estérifiant progressivement et partiellement les groupes acides. Ainsi, si l'on intercale entre chaque monomère acrylique ionisé 5 monomères non ionisables (amide acrylique), le degré de dissociation devient celui du monomère: il passe de 0.02 à 0.04 (pour l'acide) et de 0.40 à 0.87 (pour le sel de Na). Ainsi, ce n'est pas uniquement le nombre des groupes ionisables dans la macromolécule qui fixe le degré d'ionisation, mais aussi leur distance mutuelle.

L'influence de cette distance peut devenir de beaucoup la plus importante. Si l'on substitue au sel de sodium le sel d'argent, les chiffres deviennent, à la même concentration molaire (en unité des groupes ionisables, soit 0.01 M):

Propionate d'argent (monomère)	$\alpha = 0.75$
Polyacrylate d'argent	$= 0.037$
Copolymère d'argent	$= 0.43$

On voit que la degré de dissociation du polymère est 1550 fois plus petit que celui du monomère, mais que l'écartement des groupes ionisés l'augmente 230 fois, de sorte qu'il n'est plus que 7 fois plus petit que celui du monomère.

Cette propriété spécifique de l'argent semble due à une chélation par formation d'une "liaison argent", analogue à la liaison hydrogène et reliant des groupes voisins.

Une chélation analogue semble se produire avec les roussinates et on pourrait expliquer que les roussinates des polyamines ont une insolubilité de beaucoup plus grande que les roussinates des monoamines; ceux de lysine, d'éthylamine ont une solubilité 1/350 et 1/250, tandis que les chiffres s'abaissent à 1/2300 pour l'éthylènediamine, à 1/12,000 pour la cadavérine (tetraméthylène-diamine) et à 1/175,000 pour l'arcaïne (tetraméthylène-diguanidine). Corrélativement, la constante de dissociation, dans les rares cas où elle a pu être mesurée, est beaucoup plus petite aussi: égale à 0.8 pour le roussinate d'éthylamine (à 0.001 M), elle n'est plus pratiquement mesurable pour la polyvinylamine. De même, le roussinate de histidine est soluble, tandis que celui de histidyl-histidine est très peu soluble.

L'action du NSF sur les enzymes est ainsi plus facile à comprendre si les groupes NH_2 qui leur donnent leur activité sont voisins les uns des autres. S'ils étaient isolés, la petitesse de constantes K_i serait inexplicable.

En résumé on peut dire que de tous les groupes fonctionnels des protéines, seul la fonction amine et le groupe NH de l'imidazole peuvent lier le NSF et que l'un des groupes actifs des enzymes, qui sont sensibles à ce réactif, est le groupe aminé ou imidazole. Il est probable de plus que plusieurs de ces groupes doivent être voisins.

Dans les réactions enzymatiques, dans lesquelles le NSF est compétitif avec le substrat (carboxylase, alcool-déshydrogénase, lactico-déshydrogénase), ce substrat (acide pyruvique, DPN) se fixe sur les groupes aminés. Dans les cas, où le NSF agit comme un inhibiteur de couplage, il est probable que les groupes aminés sont au voisinage immédiat du lieu de fixation du substrat qui est couplé, afin que le NSF puisse se lier simultanément au substrat et à l'enzyme et empêcher ainsi leur séparation.

Naturellement, s'il existe un autre groupe actif, tel que SH, et si ces deux groupes actifs sont des sites de fixation de deux substrats, le groupe SH doit être au voisinage des groupes NH_2.

Voici deux exemples pour illustrer ce voisinage:

(a) J'ai trouvé que le *p*-chloromercuri-benzoate (PCMB) agit comme un inhibiteur de couplage pour l'acide pyruvique dans le cas de la lactico-déshydrogénase*. Comme d'après nos résultats (voir le premier mémoire), l'acide pyruvique se fixe sur les groupes aminés, le NSF étant compétitif avec ce substrat, le groupe SH doit être voisin du groupe aminé sur cet enzyme.

(b) Pour l'alcool déshydrogénase, BARRON ET LEVINE[11] sont arrivés à une conclusion analogue, en partant de l'observation que le groupe SH est protégé par les deux substrats (l'alcool et le DPN^+) contre l'inhibition par le PCMB. Ils en concluent que le groupe SH est au voisinage immédiat du système qui réagit. D'après nos résultats, le DPN se fixe sur les groupes aminés de l'alcooldeshydrogénase (puisque le NSF est compétitif avec ce substrat). En combinant ces deux résultats, on arrive à la conclusion que le groupe SH est de nouveau au voisinage du groupe aminé sur l'enzyme. Pour une autre déshydrogénase, la glycéroaldéhyde-3-phosphore-déshydrogénase, RACKER[12] admet aussi le voisinage du lieu de fixation du groupe SH.

Pour ce qui est du mécanisme d'inhibition par couplage, dont le NSF donne trois exemples décrits dans le premier mémoire, on ne peut actuellement donner que des hypothèses entre lesquelles l'expérience ne permet pas encore de choisir. Mais de toute manière, on peut la rapprocher de la constitution électronique très particulière du NSF.

Action inhibitrice des autres réactifs spécifiques des groupes aminés

Nous connaissons déjà plusieurs réactifs spécifiques de la fonction aminée: reineckate, flavianates, ruffianates, chloroaurates, chloroplatinates. Si nos conclusions relatives au NSF sont exactes, ces sels doivent agir aussi comme inhibiteurs dans les mêmes réactions enzymatiques.

Le Tableau II donne les résultats concernant l'inhibition de la carboxylase et de l'aldolase cristallisée par ces divers réactifs. Je ne donne pas de chiffres relatifs à la lacticodéshydrogénase et à l'hexokinase qui sont à peu près les mêmes.

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* En incubant l'enzyme avec PCMB pendant 15 min d'après la technique de NEILANDS[10].

TABLEAU II

INHIBITION DE LA CARBOXYLASE ET DE L'ALDOLASE

Inhibiteur	*Carboxylase*		*Aldolase*	
	conc. d'inhibiteur *M*	*inhibition* *%*	*conc. d'inhibiteur* *M*	*inhibition* *%*
NSF	$1 \cdot 10^{-6}$	33	$2.4 \cdot 10^{-5}$	21
	$2.06 \cdot 10^{-6}$	66	$4.8 \cdot 10^{-5}$	80
	$3.5 \cdot 10^{-6}$	100		
Ruffianate de Na	$3.3 \cdot 10^{-4}$	50	$1.67 \cdot 10^{-4}$	0
	$1.5 \cdot 10^{-3}$	80	$2.6 \cdot 10^{-3}$	23
			$3.0 \cdot 10^{-3}$	45
Sel de Reinecke	$7.3 \cdot 10^{-4}$	30	$1.75 \cdot 10^{-4}$	5
	$1.2 \cdot 10^{-3}$	62	$2.7 \cdot 10^{-3}$	23
			$3.0 \cdot 10^{-3}$	32
Flavianate de Na	$3.0 \cdot 10^{-3}$	0	$2.0 \cdot 10^{-4}$	0
			$2.2 \cdot 10^{-3}$	12
			$3.3 \cdot 10^{-3}$	18
Na_2PtCl_6	$1.2 \cdot 10^{-3}$	30		
	$7.2 \cdot 10^{-3}$	90		
$NaAuCl_4$	$6.8 \cdot 10^{-4}$	18		
	$2.0 \cdot 10^{-3}$	30		
	$6.8 \cdot 10^{-3}$	83		

On voit que les sels précipitant les amines sont effectivement des inhibiteurs, mais qu'ils doivent être pris à des concentrations 100 à 200 fois plus grandes que le NSF. Celui-ci a donc une activité bien supérieure.

Corrélativement, les roussinates ont des solubilités différentes de celles des reineckates, flavianates *etc*. Les derniers sont beaucoup plus hydrophiles et leur coefficient de partage entre l'eau et un liquide organique est presque infini en faveur de l'eau, juste l'inverse de ce qui se produit pour les roussinates. On comprend ainsi que le NSF soit beaucoup mieux fixé par l'enzyme qui agit comme un solvant organique, par l'intermédiaire de ses groupes basiques. Il est aussi un meilleur précipitant. Si l'on étudie la précipitation du sulfate de protamine et des produits de son hydrolyse trypsique, on constate que la précipitation de la protamine est totale par le NSF, le flavianate et le ruffianate de Na (nulle par le reineckate), mais que les peptides, contenant plusieurs restes d'arginine, précipités en milieu neutre par le NSF, ne le sont plus qu'en milieu fortement acide par le ruffianate et pas par le flavianate, ni par le reineckate.

Une théorie générale de la solubilité permetterait sans doute de mettre en rapport cette propriété du NSF avec sa constitution. Malheureusement, nous ne la possédons pas actuellement.

Sur le nombre de sites actifs et la structure secondaire

Les résultats qui précèdent sont en faveur de l'hypothèse souvent formulée, d'après laquelle la structure secondaire de la molécule joue un rôle important dans l'activité des enzymes.

En effet, bien que la simple séquence des acides aminés puisse amener des sites actifs à être voisins, une cause plus probable est une structure secondaire, qui rapproche des points éloignés de la chaîne peptidique. La probabilité pour qu'ils soient voisins dans cette chaîne étendue a une valeur appréciable si leur nombre est réduit à deux. Mais elle devient négligeable s'il est égal au moins à quatre, comme pour la lactico-deshydrogénase (au moins deux groupes NH_2, un SH et un groupe indéterminé qui fixe le DPNH).

PARTIE EXPÉRIMENTALE

Détermination du coefficient de partage. J'ai employé comme phase organique liquide le butanol secondaire et comme phase solide la soie naturelle. Les mesures ont été faites par colorimétrie du NSF. Le coefficient de partage dépend de la concentration ionique de la phase aqueuse. Elle doit être amenée à la même valeur dans le liquide de comparaison (par addition, par exemple, de malate de Na) que dans la solution du corps à fonction thiol (par exemple, de thiomalate de Na).

Réactivité des groupes SH. Si le groupe SH était modifié par le NSF ou s'il entrait dans une combinaison peu dissociée avec ce sel, sa réactivité serait modifiée. J'ai choisi comme test la réaction du glutathion avec les sels de cadmium. Le glutathionate de cadmium est assez soluble et pour que sa précipitation soit intégrale, il faut des conditions strictes, établies par BINET ET WELLER[13]. La précipitation ne serait pas intégrale si le groupe SH du glutathion était lié au NSF et serait nulle s'il était modifié. Or elle est totale en présence d'un excès de NSF. Le glutathion oxydé (forme S–S) ne précipite pas par le lactate de cadmium en présence, comme en absence de NSF. Ce sel ne réduit donc pas les ponts S–S.

Equilibre de membrane. Les membranes utilisées étaient soit des tubes en cellophane (Nojax, Visking Co), soit des sacs en collodion. Pour chaque polymère on a effectué deux séries d'expériences: dans l'une le NSF a été ajouté dans l'eau, à l'extérieur de la membrane, dans l'autre, il a été mélangé à la solution du polymère. L'équilibre a été atteint généralement au bout de 2 à 3 semaines. (à 0° et à l'obscurité). La concentration de chaque phase en NSF a été déterminée colorimétriquement.

Polymères. L'acide acrylique et *l'acool polyvinylique* commerciaux ont été soumis à un fractionnement et la fraction de poids moléculaire environ 200,000 a été employée. *La polyacrylamide* et *la polyméthylol-acrylamide* ont été obtenues par la polymérisation des monomères avec un système redox. Les deux monomères ont été gracieusement fournis par la Cyanamid Co, à laquelle nous adressons nos remerciements. *La polyvinylpyrrolidone* provenait de la Badische Anilin und Soda Fabrik, Ludwigshafen, et a été fournie à titre gracieux par MM. le prof. Dr. HOPFF et le Dr. FIKENTSCHER, à qui je dois mes remerciements. *La polyvinylamine* a été donnée par MM. D. D. RAYNOLDS et M. HUGGINS de la Eastman-Kodak Co. Je les remercie pour leur don précieux, sans lequel cette étude aurait été impossible.

Inhibition par divers réactions spécifiques des groupes aminés. Les enzymes et le mode de détermination de leur activité sont décrits dans le premier mémoire[1].

RÉSUMÉ

Le mémoire précédent[1] a montré que le sel de Roussin ou nitrososulfure de fer (NSF) a une action inhibitrice très forte sur une série d'enzymes. Il est souvent compétitif avec le substrat.

Pour déterminer la nature chimique du site actif de l'enzyme, il faut connaître la fonction chimique qui réagit réversiblement avec l'inhibiteur. De tous les groupes fonctionnels connus des protéines, seules les fonctions aminés et NH de l'imidazole peuvent se combiner au NSF, en donnant un sel dissociable. La solubilité, ainsi que la constante de dissociation des combinaisons obtenues avec les diverses amines, diminuent avec l'augmentation du nombre de groupes aminés par molécule et surtout avec leur rapprochement mutuel. Ceci laisse entrevoir la possibilité d'une chélation.

Pour pouvoir expliquer les faibles valeurs des K_i obtenues pour les enzymes étudiées, il faut admettre que le site actif des enzymes, sur lequel se fixe le NSF, est constitué par plusieurs groupes aminés, très rapprochés les uns des autres. Ils seraient le site de fixation du DPN^+ et de l'acide pyruvique (dans le cas de la carboxylase, de l'alcool-déshydrogénase et de la lactico-déshydrogénase). Les sites de fixation de l'alcool (alcool-déshydrogénase), de l'ATP (hexokinase) et du fructose-diphosphate (aldolase) semblent être au voisinage immédiat de ces groupes aminés, puisque le NSF, fixé sur ces groupes, provoque une inhibition de couplage par rapport à ces substrats. Dans deux cas, l'alcool-déshydrogénase et la lactico-déshydrogenase, le groupe SH, qui est le second groupe actif, est au voisinage immédiat des groupes basiques.

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A SPECTROPHOTOMETRIC STUDY OF THE SPONTANEOUS AND MICROSOME-INDUCED SWELLING OF RAT-LIVER MITOCHONDRIA

Y. AVI-DOR

Israel Institute for Biological Research, Ness-Ziona (Israel)

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SUMMARY

The effect of microsomes on the swelling of rat liver mitochondria has been studied. A kinetic study of the changes in the optical density of the mitochondrial suspension during swelling revealed that the swelling is preceded by a lag. Addition of microsomes shortens this lag period, but otherwise the microsome-induced swelling resembles closely spontaneous swelling. The duration of the lag depends on the concentration of mitochondria, pH, temperature and osmotic pressure. The active component of the microsomes has the properties of a fatty acid. Indeed, fatty acids have been shown to produce the same effect. Some aspects of the spontaneous and the microsome-induced swelling are discussed.

INTRODUCTION

Mitochondria isolated from an isotonic sucrose homogenate swell in a hypotonic sucrose medium in accordance with the osmotic law[1]. However, there exists some doubt whether the whole mitochondrion responds to the osmotic pressure or only an inner compartment which is relatively impermeable to sucrose[2,3,4]. The almost instantaneous adjustment of mitochondria to the prevailing osmotic pressure is followed by a slower phase of swelling due to the penetration of sucrose into an internal compartment[5,6]. This so-called spontaneous swelling takes place even in an isotonic sucrose solution and is accompanied by an activation of the latent mitochondrial ATPase[7], release of mitochondrial ATP[8] and other autolytic changes. There is also a strong indication that the permeability of the inner membrane to sucrose increases during the slow phase of swelling[9].

A variety of compounds is known to accelerate the structural deterioration of mitochondria[10]. Some of them increase the rate of the spontaneous swelling[9] while others seem to act by a different mechanism[11]. Of special interest is the study of the effect on the swelling of normal cell constituents likely to contaminate the mitochondrial cell fraction. Such constituents are the microsomes which adhere tenaciously to mitochondria[12] and affect various mitochondrial activities[13]. It is shown in the present investigation that addition of microsomes accelerates markedly the swelling in isotonic sucrose. This microsome-induced swelling, like the effect of thyroxine[9], is essentially a stimulation of the spontaneous swelling.

EXPERIMENTAL

Particle preparations

Mitochondria. Rat liver and kidney mitochondria were prepared by the method of SCHNEIDER[14], as modified by SWANSON[15], rat heart sarcosomes as described previously[16], and rat brain mitochondria by the procedure of BRODY AND BAIN[17].

Microsomes were separated as described previously[16] and washed once with distilled water.

Alcohol–ether extract of microsomes

Freeze-dried microsomes (1 g) were refluxed for 2 h with 200 ml of an ethanol (3 vol.)–diethyl ether (1 vol.) mixture and filtered. The volume of the filtrate was reduced to 2 ml *in vacuo*. Ice-cold acetone (20 ml) was added and the mixture left overnight at 0°. The precipitate formed was removed by centrifuging (acetone-insoluble fraction) and the supernatant was evaporated to dryness in a stream of nitrogen. An aliquot of the dry residue was hydrolyzed with a 20 % alcoholic potassium hydroxide solution for six hours. The free fatty acids and the nonsaponifiable material were separated by the usual procedure. To assay the activity of the various fractions of the microsomal extract or of glycerides and fatty acids, a 1 % solution of the lipid was prepared in ethanol. This stock solution was diluted 100 fold with water and sonicated in a Raytheon sonic oscillator of 10 kc for 15 min on the day of the experiment.

Methods of assay

The standard assay medium for the spectrophotometric experiments contained 0.02 *M* Tris buffer of pH 7.4, 0.25 *M* sucrose, and mitochondria in an amount to give an optical density of 0.500 at 600 mμ in the Beckman spectrophotometer (Corex or Pyrex cells of 1 cm light path). For liver mitochondria, this amount is equivalent to 30 μg N/ml. The turbidity was measured against a blank containing all the components with the exception of mitochondria. As a rule, experiments were conducted at room temperature (16–18°). When the effect of temperature was investigated, the cells were kept in a water bath at the required temperature, except during the time of measurement (approx. 20 sec). Viscosity was measured in an Ostwald viscosimeter at 18°.

Chemicals

Lecithin was prepared according to HANAHAN[19]. Other chemicals were obtained from the following commercial sources: glycerides and fatty acids, BDH; crystalline ATP, Sigma; *d,l*-thyroxine, Mann Research Laboratories; crystalline bovine serum albumin, serum globulin and ovalbumin, Armour Lab., dialysed for 48 h against 0.002 *M* Tris buffer of pH 7.4; cellulose methyl-ether (methocel), Dow Company.

RESULTS

Comparison of the kinetics of the spontaneous and of the microsome-induced swelling

The kinetics of optical density changes which occurred in rat liver mitochondria, suspended in buffered 0.25 *M* sucrose, are presented in Fig. 1. It is apparent that a lag preceded the spontaneous swelling which was shortened on the addition of microsomes. After the completion of the swelling, the final level of optical density was the same, irrespective of whether microsomes were added or not. By increasing the amount of microsomes added, the lag could be suppressed almost completely. The kinetics of swelling in isotonic KCl or NaCl were essentially the same as in sucrose, but the duration of the lag was shorter.

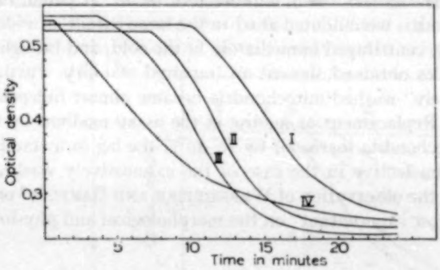

Fig. 1. Kinetics of spontaneous and microsome-induced swelling. Standard assay medium as described in EXPERIMENTAL. Temp. 18°. Curve I: spontaneous swelling; Curves II, III and IV: 10 µg, 20 µg, 200 µg (dry weight) of microsomes added/ml of medium.

The relative sensitivity of mitochondria obtained from various organs to the microsomal effect decreased in the following order: liver > kidney ≫ heart > brain. This order of sensitivities was observed also in the case of the spontaneous swelling and is similar to that reported by TAPLEY AND COOPER for thyroxine-induced swelling[20]. Microsomes prepared from kidney had about half the effect of liver microsomes when compared on the basis of dry weights.

The influence of the reaction conditions on the kinetics of the spontaneous and microsome-induced swelling

(a) *Osmotic pressure.* Under the standard conditions of the experiment, 0.25 *M* sucrose was used for the maintenance of the osmotic pressure. When the concentration of the sucrose was varied between 0.03 *M* and 0.25 *M*, only the initial optical density changed but the duration of the lag remained almost constant (Table I). In a sucrose concentration higher than 0.25 *M*, the lag became longer. The latter increase in stability is not connected with the higher viscosity of the medium, since the viscosity

TABLE I

EFFECT OF SUCROSE CONCENTRATION ON THE DURATION OF THE LAG

0.02 *M* Tris buffer, pH 7.4 and sucrose in various concentrations. 20 µg/ml (dry weight) of microsomes has been added when indicated. Temperature 18°.

Concn. of sucrose (M)	*Initial optical density*	*Duration of lag (in minutes)*	
		Spontaneous	*Microsomes added*
0.50	0.406	24.0	18.0
0.25	0.420	11.0	5.5
0.10	0.359	11.5	6.5
0.03	0.250	12.0	6.5

of the isotonic sucrose could be increased tenfold by the addition of 0.05 % methocel solution without affecting the duration of the lag phase.

(b) *Concentration of mitochondria.* The duration of the lag phase decreased markedly with dilution of the mitochondria, while the effect of the microsomes depended on the ratio of the two cell fractions (Table II). The considerable influence of the dilution suggested the loss of a protective factor. It could, indeed, be shown that when mitochondria were diluted at 0° to the same extent as under the conditions of the optical assay, centrifuged immediately in the cold, and brought to the original volume, the particles obtained showed an impaired stability. Furthermore, the lag of such "exhaustively" washed mitochondria became almost independent of concentration (Table II). Replacement of sucrose in the assay medium by the supernatant of the washed mitochondria increased by 20–40 % the lag in a standard preparation but proved to be ineffective in the case of the exhaustively washed particles. The latter results recall the observation of WERKHEISER AND BARTLEY[3] on the preserving effect of their "sucrose supernatant" on the morphological and physiological integrity of mitochondria.

TABLE II

EFFECT OF THE CONCENTRATION OF MITOCHONDRIA ON THE DURATION OF THE LAG

Standard assay medium. Temp. 18°. "Exhaustively" washed mitochondria were prepared as described in the text. 20 μg/ml (dry weight) of microsomes or 1 m*M* ATP or 1 m*M* KCN was added as indicated.

Concn. of mitochondria (μg/ml)	*Duration of lag (in minutes)*				
	Standard mitochondria		*"Exhaustively" washed mitochondria*		
	Spontaneous	*Microsomes added*	*Spontaneous*	*ATP added*	*KCN added*
10	3.5	1.0	—	—	—
20	5.0	2.0	—	—	—
40	7.5	4.5	1.5	8.0	10.0
80	14.0	12.0	—	—	—
160	20.0	—	2.0	10.0	12.0
200	29.0	—	—	—	—

pH. In the range between pH 6 to pH 8 the lag period of the spontaneous swelling as well as that of the microsome-induced swelling decreased with increasing pH. The shortening of the lag caused by the addition of microsomes became more pronounced the higher the pH was. According to HARMAN AND KITIYAKARA[21], mitochondria increase their size with increasing pH, and concomitantly their structure is loosened up. Such opening up of the structure could increase the number of sensitive sites accessible for the action of the microsomes.

(c) *Temperature.* LEHNINGER *et al.*[9] investigated the effect of temperature on the time required to reach one fourth of the maximum extent of swelling. The Q_{10} was found to be very high and showed a maximum at 20°. Since during the present investigation it became apparent that the duration of lag is a highly characteristic parameter of mitochondrial swelling, its temperature dependence was studied. The reciprocal value of the duration of the lag was taken as the overall rate. The value of 100 was assigned arbitrarily to the "rate" at 32° in the absence of microsomes and the logarithm of the relative velocities was plotted against $1/T$ (Fig. 2). From the slope of this Arrhenius plot the apparent energy of activation was calculated by the usual method. The values obtained ranged between 19,000 and 21,000 cal/"mole" for five different batches of mitochondria. Addition of microsomes which shortened the lag period, and of ATP which stabilized the mitochondria (see below), were found to affect merely the position, but not the slope of the plot.

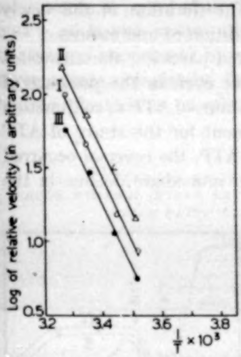

Fig. 2. Arrhenius plot for the lag phase. Curve I: spontaneous swelling; Curve II: 20 μg/ml microsomes added; Curve III: 0.33 mM ATP added. Standard reaction medium.

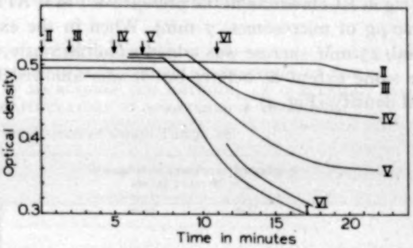

Fig. 3. Effect of the time of addition of KCN. Standard medium. Temp. 18°. Experimental points before the addition of KCN not indicated. Curve I: No KCN added; Curves II, III, IV, V, VI: 1 mM KCN added at the time indicated by an arrow and the number of the respective curve.

Effect of KCN and ATP on spontaneous and microsome-induced swelling

A great variety of compounds is known to confer increased stability upon the mitochondria[9, 10]. Of special interest among them is KCN, which presumably affects the state of oxidation of the respiratory carriers and of ATP, which is able not only to stabilize intact mitochondria but also to reverse the swelling of mitochondria under certain experimental conditions[9, 22, 23]. In order to localize the phase in which the damage to the osmotic barrier occurs, the effect of the time of addition of the stabilizing agents in relation to the protection attained has been investigated. It can be seen from Fig. 3 that when the addition of KCN was made during the lag phase its effect manifested itself both in an elongation of the lag of spontaneous swelling and in a higher final level of optical density. Addition at the end of the lag or during the decline in optical density affected only the final level, but not the lag. The sooner the addition was carried out, the greater was its effect. Quite similar results were obtained when ATP was added at various phases of the swelling process. The pattern of action of the two stabilizers on the microsome-induced swelling was qualitatively the same as on the spontaneous swelling and for the sake of brevity will not be described here. The exhaustively washed mitochondria still responded to the action of KCN and ATP. 1 mM KCN or 1 mM ATP was able to increase the lag from 2 to approx. 10 min.

Effect of microsomes on "reconstituted" mitochondria

The effect of microsomes was also investigated on mitochondria which were once swollen but then contracted under the influence of added ATP. These experiments were conducted in a 0.15 *M* KCl suspension of mitochondria since according to LEHNINGER *et al.* no reversal takes place in isotonic sucrose[9]. In accordance with the observation of LEHNINGER *et al.*[9], a partial reversal of the decline in light absorption took place when ATP was added to the test system, after the swelling had proceeded already to a considerable extent (Fig. 4). The duration of the newly established lag phase was unaffected, however, by the addition of microsomes.

In contrast to the lack of response of the reconstituted particles, the unswollen mitochondria remained sensitive to the microsomal effect even in the presence of ATP (lag in KCl medium in the presence of 1 m*M* ATP: 12 min; of ATP in conjunction with 20 µg of microsomes: 7 min). When in the experiment for the study of ATP reversal, 25 m*M* sucrose was added simultaneously with ATP, the reversal occurred to the same extent as before, but it was followed by an immediate decline in the optical density (Fig. 4).

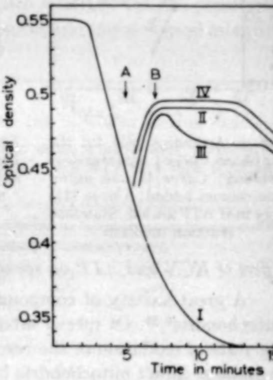

Fig. 4. Reversal of swelling by ATP (1 m*M*) and the effect of microsomes (20 µg dry weight/ml) and of sucrose (25 m*M*) upon it. Assay medium: 0.02 *M* Tris, pH 7.4, KCl, 0.15 *M*. Curve I: No addition made; Curve II: ATP added at the time indicated by arrow "A"; Curve III: ATP + sucrose added simultaneously at time "A"; Curve IV: ATP added at time "A" and microsomes at time "B".

Effect of microsomal and other lipids on swelling

The swelling-inducing activity of microsomes was found to be insensitive to heating at 100° for 15 min. Sonication increased their activity. After refluxing the microsomes with a mixture (3:1) of ethanol and ether, all the activity was found in the extract. The activity of the acetone-soluble material was greater than that of the acetone-insoluble substance. Removal of the non-saponifiable material from the acetone-soluble fraction left the largest part of the active principle behind (Table III). Since isolated microsomes are known to contain large amounts of free fatty acids[13] and since, as shown in Table III, a number of fatty acids are able to stimulate swelling to a similar extent as does the microsomal extract, it was unlikely that the microsomal activity could be connected with a single specific substance. No further fractionation was attempted therefore. It can be seen from Table III that free fatty acids are more active than glycerides. Most active are unsaturated acids, and among the saturated ones those of intermediate chain length. These results compare well with those of PRESSMAN AND LARDY on the activation of the latent mitochondrial ATPase by an acetone extract of microsomes and by free fatty acids[13]. The effects described by these authors are probably manifestations of microsome-induced deterioration of the mitochondrial structure.

Since serum albumin is known to bind free fatty acids with great affinity[21], its effect on the stimulation of swelling caused by microsomes, by the acetone–ether extract and by palmitic acid was compared. The concentration of the stimulants was chosen in such a manner that they shortened the lag by approx. 50 % (20 μg/ml of microsomes, 6 μg/ml of alcohol–ether extract, and 0.005 m*M* of palmitic acid). When 0.01 % of bovine serum albumin was added to the system, it increased the lag in the control experiment by 15–20 % while it suppressed the accelerating action of the three agents completely. On the other hand, 1 % of serum globulin or ovalbumin had no effect either on spontaneous or on microsome-induced swelling.

TABLE III

COMPARISON OF THE EFFECTIVENESS OF VARIOUS SUBFRACTIONS OF THE CRUDE ETHANOL–ETHER EXTRACT OF MICROSOMES (see METHODS), OF GLYCERIDES AND OF FREE FATTY ACIDS AS STIMULATORS OF SPONTANEOUS SWELLING

Standard conditions of assay. Temp. 18°.

Fraction or substance added	*Concentration causing a shortening of the lag phase by 50 %*	
	μg/ml	*mM*
Microsomes	20	
Sonicate of microsomes	10	
Crude extract	6.6	
Acetone-soluble fraction	1.8	
Acetone-insoluble fraction	6.3	
Non-saponifiable material	6.0	
Free "fatty acid" fraction	0.8	
Tristearate		0.2
Trioleate		0.1
Lecithin		0.1
Caprylic acid		0.01
Caproic acid		0.01
Lauric acid		0.003
Myristic acid		0.002
Palmitic acid		0.005
Stearic acid		0.007
Oleic acid		0.0015
Linoleic acid		0.0015

DISCUSSION

For an interpretation of the spectrophotometric data obtained in the present study in terms of an osmotic process some assumptions have to be made in accordance with recent views on spontaneous swelling. These assumptions are:

(a) the existence of an inner mitochondrial compartment, impermeable to sucrose, in the freshly isolated particles (*cf.* ref. [3–6, 9]); (b) a connection between the state of oxidation of some constituents of the membrane and permeability (*cf.* ref. [9]).

There are a number of observations, such as the effect of dilution or exhaustive washing on the duration of the lag, which indicate that during the process of isolation protective substances are removed from the mitochondria. Since KCN or ATP were found to have no effect on the lag, once the swelling started, but were still effective in the washed preparation, one must assume that the latter is only sensitized but nevertheless still possesses an intact osmotic barrier.

The magnitude of the energy of activation (19,000–21,000 cal./"mole") calculated for the hypothetical reaction which takes place during the lag phase, is compatible with a biological oxidation process dependent on consecutive enzyme reactions (*e.g.* 16,000 cal./"mole" for succinoxidase[25]). The elongation of the lag by KCN could thus be due to the inhibition of the oxidative process. ATP presumably effects the lag by a different mechanism since it is able under certain conditions *to reverse* the swelling. Most probably it exerts a direct stabilizing effect on the structure of the membrane, as suggested by RAABLAUF and co-workers[8, 26]. There exists, however, an intimate relationship between the factor controlled by ATP and the one regulated by the oxidation process since the same energy of activation was found for spontaneous or microsome-induced swelling and for the swelling in the presence of ATP. Such interrelation could be visualized by assuming the existence of a *contractile principle* in the mitochondrial membrane, as it has been pointed out already by RAABLAUF[26]. The contractile unit is kept in a contracted state when sufficient ATP is present, but unfolds when this stabilizer is lost (*e.g.* by the action of surface-active agents like free fatty acids originating from microsomes). The unfolding exposes sensitive groups, which regulate the permeability, to oxidation. The change in permeability and—as a corollary—swelling can thus be avoided either by keeping the membrane in its contracted state (ATP) or by inhibiting the oxidation of the sensitive groups (anaerobiosis[27], KCN, other inhibitors of respiration[9]).

The lag is terminated by a transitory phase in which addition of KCN or ATP is unable to stop the decline in the optical density, but still has an effect on the final level of the turbidity reached. WERKHEISER AND BARTLEY[3] considered the possibility that the mitochondrial population is not homogenous osmotically. Differences might exist between one mitochondrion and another also with regard to their state of oxidation. Therefore, a part of the mitochondria might have reached the critical state of oxidation before the others and will no longer respond to the stabilizers. The non-responding fraction will be larger the later the stabilizers are added and the final optical density will be correspondingly lower.

The elucidation of the partial reversal of the decrease in the optical density in KCl suspensions of mitochondria[9] by ATP requires further experiments. The results shown in Fig. 4 indicate that the once-swollen mitochondria did not regain their former relative impermeability to sucrose. They differ also from the unswollen particles in their lack of response to microsomes.

In a hypertonic sucrose solution, the lag is considerably extended. Probably extensive shrinkage of the mitochondria reduces the number of sites which are exposed to the influence of deleterious factors. Since, according to HARMAN AND KITIYAKARA[21], mitochondria contract also in an acid medium, the increased stability at a lower pH might have a similar explanation. It seems, however, that already in 0.25 M sucrose all the sensitive sites are bared and therefore additional stretching of the membrane under hypotonic conditions will not reduce further the duration of lag phase.

Since no qualitative differences were found between microsome-induced and spontaneous swelling, it is not possible yet to evaluate the part played by contaminating microsomes in the so-called spontaneous swelling. The microsomal fraction originates mainly from the cytoplasmic system of membranes and granules described as endoplasmic reticulum[28]. In view of the intimate contact which exists between the reticulum and mitochondria[28], the possibility has to be considered that the reticulum influences the permeability of the mitochondrial membrane also in the cell. The main vehicles for the microsomal effect seem to be free fatty acids which were liberated from the reticulum as a result of the action of esterases. Owing to their surface activity, fatty acids are likely to stimulate the unfolding of the contractile unit in the membrane. Fatty acids and especially those of intermediary chain-length were shown to denature ovalbumin[29]. While the largest part of the fatty acids found in the microsomes is liberated only during the procedure of isolation[13], even the much smaller amount which might occur in the intact cell could modify the mitochondrial structure.

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STUDIES ON THE NUCLEOTIDE ARRANGEMENT IN DEOXYRIBONUCLEIC ACIDS

III. IDENTIFICATION OF METHYLCYTIDINE DERIVATIVES AMONG THE ACID DEGRADATION PRODUCTS OF RYE GERM DNA

HERMAN S. SHAPIRO AND ERWIN CHARGAFF

Cell Chemistry Laboratory, Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y. (U.S.A.)

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SUMMARY

This paper reports the isolation from an acid hydrolysate of rye germ deoxyribonucleic acid of three derivatives of methylcytidine, *viz.*, the 3′,5′-diphosphate and the two dinucleoside triphosphates that this pyrimidine nucleoside shares with cytidine and thymidine, respectively. Some of the properties of these compounds are also described.

INTRODUCTION

In addition to the four usual nitrogenous constituents of deoxyribonucleic acids[1] 5-methylcytosine occurs as a minor component in nucleic acids of animal origin[2] and as a considerable component in those isolated from plant sources[3]. In microbial deoxyribonucleic acids this pyrimidine has not been found[1], although 5-methylcytosine has recently been claimed to be present in the ribonucleic acid of *E. coli*[4]. By far the highest concentration of 5-methylcytosine, about 6 mole % of the nucleotides, is encountered in the deoxyribonucleic acids of cereal germ[1,3,5].

When complex mixtures of fragments produced by the acid hydrolysis of deoxyribonucleic acids of animal origin were analyzed through separation on ion exchange columns, the appearance of deoxymethylcytidine 3′,5′-diphosphate in front of the corresponding diphosphate of deoxycytidine was inferred only indirectly on spectroscopic grounds[6,7], but, in another instance, demonstrated by identification[8].

The study of certain aspects of the nucleotide arrangement by means of the differential distribution analysis of pyrimidine nucleotides[9-11] has, in the following communication[12], been extended to the deoxyribonucleic acid of rye germ. Before such studies could be undertaken, a better characterization of deoxymethylcytidine 3′,5′-diphosphate, as well as of other derivatives of 5-methylcytosine found among the products of acid hydrolysis, but not identified previously, was required; and this is provided in the present paper.

EXPERIMENTAL*

Procedures and material

The analytical methods and most of the other procedures employed have been outlined before[10]. For ion exchange chromatography, the anion exchanger Nalcite SAR (Dowex-2), 8 % cross linkage, 100–200 mesh, was used. Filter paper chromatography was carried out on Whatman paper No. 1 with one of the following solvent systems: buffered isobutyric acid–isobutyrate[14], isopropanol–NH_3[15], isopropanol–HCl[8], and *n*-butanol–0.1 *N* NH_3 (6:1, v/v)[5].

The preparation of DNA from rye germ that served as the starting material is described in the following paper[12]. Reference preparations of M and pM were supplied by the California Corporation for Biochemical Research, Los Angeles, California.

Isolation of deoxymethylcytidine derivatives

After the hydrolysis of 560 mg of DNA with 15 ml of boiling 0.2 *M* H_2SO_4 for 2 h under a reflux the solution was freed of inorganic acids by adjustment to pH 5.5 with a saturated aqueous solution of $Ba(OH)_2$ and centrifugation. The clear supernatant solution, brought to pH 10 with aqueous ammonia, was placed on a Dowex-2-formate column. The pertinent portion of the gradient elution pattern is reproduced in Fig. 1. The identification of the separated fractions was in part based on the characteristics of the previously described elution patterns[10], [11].

Characterization of deoxymethylcytidine derivatives

The pMp and pCp fractions were, before isolation, further purified by rechromatography on Dowex-2. These eluates as well as the other five fractions collected (Fig. 1)

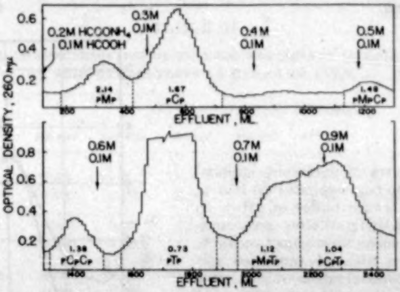

Fig. 1. Concentration gradient elution pattern of acid hydrolysate of rye germ DNA. Conditions: Volume of mixing chamber, 250 ml; the arrows underneath the indicated formate buffer concentrations (upper figure ammonium formate, lower figure formic acid) denote the points of change in the contents of the single reservoir; Dowex-2 (8 ×), 100–200 mesh; 15 cm × 2.5 cm diam.; the initial concentration of buffer in the mixing chamber was 0.1 *M* $HCOONH_4$–0.1 *M* HCOOH; 10 ml fractions. The dotted lines delimit the areas of pooled fractions, with the ratios of their O.D. at 280/260 mμ placed over the designations.

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* We shall use the following abbreviations: DNA, deoxyribonucleic acid of rye germ; Py, pyrimidine deoxyribosides; C, deoxycytidine; M, deoxy-5-methylcytidine; T, thymidine; p, esterified phosphoric acid. In presenting the structures of nucleotides, nucleoside diphosphates, dinucleotides, etc., we shall use the principles enumerated previously[10], but for the sake of uniformity with the conventions used in the description of ribonucleotides[13] we shall place p at the left of the symbol if the phosphoric acid is linked to the 5′ hydroxyl of the nucleoside, at the right if it is on the 3′ hydroxyl.

were neutralized with aqueous ammonia, lyophilized and freed of ammonium formate by sublimation. Purity checks by filter paper chromatography showed only the two adjoining cuts pMpTp and pCpTp* to be contaminated with each other; they were further purified by chromatography. The relative mobilities given in Table I and the absorption spectra of the methylcytidine derivatives illustrated in Fig. 2 supplement

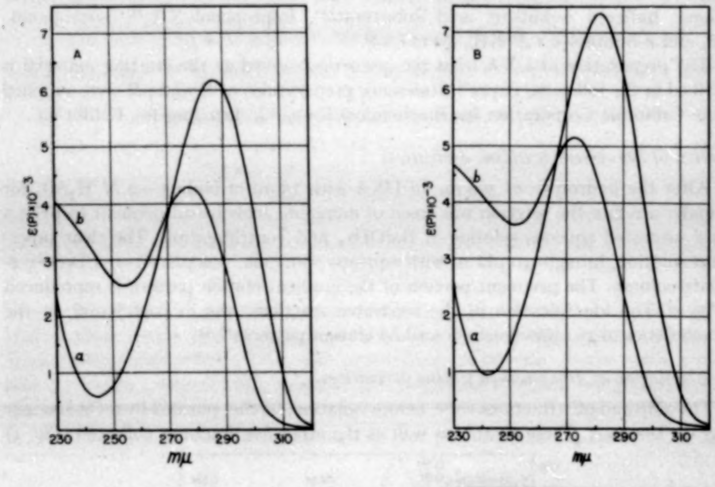

Fig. 2. Absorption spectra of nucleoside diphosphate and dinucleoside triphosphates (a) in 0.01 *N* HCl, (b) in 0.1 *M* borate buffer of pH 7. A. pMp; B. pMpCp; C. pMpTp.—After rechromatography of the fractions on filter paper, extracts of the adsorption zones with the indicated solvents served for the determination of ε(P) after correction for the absorption of similarly treated blank areas.

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[*] The dinucleoside triphosphate fractions presumably contain both sequential isomers, *e.g.*, pMpTp and pTpMp.

previously given data[10,11]. The list of extinction values in Table II is useful for the identification and estimation of the compounds under investigation.

All newly isolated methylcytidine derivatives were subjected to analysis. Samples were hydrolyzed in sealed tubes for 30 min at 175° with 88 % formic acid (about 0.1 ml of acid for 0.1 mg of substance). Portions of the hydrolysates served for P analysis and for pyrimidine estimation by paper chromatography in the *n*-butanol–NH_3 solvent. The following molar proportions were found per 1 mole of methylcytosine: (a) for pMp, 2.1 P; (b) for pMpCp, 1.1 cytosine and 3.4 phosphorus; (c) for pMpTp, 1.0 thymine and 3.0 phosphorus.

TABLE I

CHROMATOGRAPHY OF PYRIMIDINE NUCLEOSIDE DERIVATIVES OBTAINED FROM ACID HYDROLYSATES OF DNA; RELATIVE R_F VALUES*

	Buffered isobutyrate	*Isopropanol–NH_3*
pM	1.90	1.08
pCp	1.00	1.00
pMp	1.20	1.20
pTp	0.83	1.23
pMpCp	1.08	0.91
pCpCp	0.95	0.95
pMpTp	0.90	1.07
pCpTp	0.84	1.07

* With the R_F of cytidine diphosphate, pCp, taken as 1.00.

TABLE II

SPECTRAL CHARACTERISTICS OF SEVERAL FRAGMENTS ISOLATED FROM AN ACID HYDROLYSATE OF RYE GERM DNA*

Compound	*pH*	*ε(P) × 10⁻³ at indicated wavelength***					
		320 mμ	*290 mμ*	*280 mμ*	*260 mμ*	*250 mμ*	*Maximum*
pM	2	0.13	12.3	11.2	3.53	1.40	12.5 (287 mμ)
	7	0.04	5.99	8.51	5.15	4.88	8.64 (277 mμ)
pMp	2	0.06	6.06	5.64	1.89	0.80	6.18 (286 mμ)
	7	0.03	2.89	4.25	2.72	2.66	4.30 (278 mμ)
pMpCp	2	0.07	6.38	7.29	3.18	1.46	7.37 (282 mμ)
	7	0.05	2.36	4.59	4.04	3.64	5.17 (273 mμ)
pCpCp	2	0.04	5.81	7.97	4.04	1.88	8.01 (279 mμ)
	7	0.05	1.61	4.51	4.72	4.04	5.68 (271 mμ)
pMpTp	2	0.04	4.47	5.91	4.30	2.43	6.08 (276 mμ)
	7	0.03	2.18	4.50	4.70	3.70	5.47 (271 mμ)
pCpTp	2	0.01	4.09	6.45	4.94	2.74	6.90 (273 mμ)
	7	0.03	1.56	4.34	5.11	3.86	5.87 (268 mμ)

* The values are based on an average of at least three independent determinations.

** ε(P) is the atomic extinction coefficient with respect to phosphorus[16]. The value for ε, the molecular extinction, is obtained by multiplying ε(P) by the number of P atoms in the particular compound. The values in the last column include the positions of the absorption maxima in parentheses.

Kinetics of the acid hydrolysis of deoxymethylcytidine and its phosphorylated derivatives

Approximately 1 % solutions of M, pM and pMp in 0.2 *M* H_2SO_4 were employed. The hydrolysis was carried out with 0.2-ml samples at 100° under a reflux in the arrangement previously described (see p. 601 of ref. [10]). Portions of the hydrolysates were removed periodically for P analysis and chromatography, isopropanol–HCl being used for the hydrolysates of M and the isobutyrate solvent for those of pM and pMp. The results are shown in Fig. 3 for a first order reaction, as no acid is consumed and the H_2O concentration remains essentially unchanged.

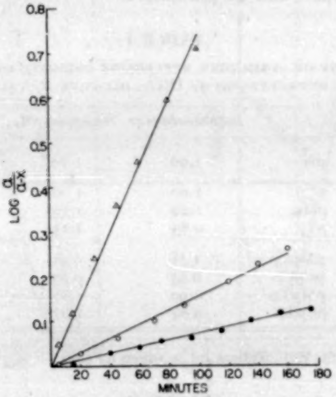

Fig. 3. First order kinetics of hydrolysis (0.2 *M* H_2SO_4, 100°) of deoxymethylcytidine △, deoxymethylcytidine 5′-phosphate ○, and deoxymethylcytidine 3′,5′-diphosphate ●.

RESULTS AND DISCUSSION

The application of the present findings to problems of DNA structure will be discussed in the subsequent paper[12]. It was obvious from our previous studies[10,11] that under the conditions of DNA hydrolysis some decomposition of the methylcytidine derivatives must take place. It could, however, be inferred that the presence of a methyl group on C-5 of the pyrimidine ring would not cause a material change in the hydrolytic rates of pMp and the other methylcytidine derivatives from the rates recorded for the corresponding compounds containing cytidine[10]. This is confirmed by the results of the acid degradation of M, pM and pMp reported here. The value of the first order rate constant, $k = 0.0017\ \text{min}^{-1}$, for pMp in 0.2 *M* H_2SO_4 at 100° is identical with that previously found for pCp[10]. Other *k* values are similarly identical or very close, namely, 0.022 min^{-1} for C, 0.017 min^{-1} for M; 0.0037 min^{-1} for pC, 0.0036 min^{-1} for pM. Information on the decay rates of these substances is necessary for the computation of correction factors to be used in the analysis of the products of the acid degradation of DNA[11,12,17].

In our previous study[10] we observed a slight hypochromic effect when the extinctions of compounds of the type p Py p or p Py p Py p were compared with the sum of the extinctions of pPy. The spectral differences found here between pMp, pMpCp or pMpTp and their respective mononucleotide components are, however, within the range of the average deviations of the individual spectra.

In addition to the spectroscopic data given in Table III, the extinction value at the isosbestic point between pH 2 and pH 7 can be used for quantitative estimation when pH adjustment is inconvenient. The $\varepsilon(P)$ values and the isosbestic points are: for pMp, 3,940 at 271 mμ; for pMpCp, 4,750 at 267 mμ; for pMpTp, 5,200 at 265 mμ (compare also the absorption spectra in Fig. 2).

The combined use of the evidence from spectroscopic and chromatographic properties and from the analytical determination of pyrimidine to phosphorus ratios has permitted the identification of an entire series of products of the acid hydrolysis of DNA having the general structure $[\mathrm{Py}]_n[\mathrm{p}]_{n+1}$[8,11,17]. Two more compounds of this type are added to this list in the present study. In the elution pattern of an anion exchange column, pMpCp and pMpTp appear just ahead of the corresponding compounds containing C instead of M. As has been pointed out before in the discussion of "solitary" and "bunched" units in DNA[11], the proportions of these dinucleoside triphosphates as well as of the nucleoside diphosphates must, under the degradative conditions followed here, closely reflect the number of such units that, in the sequence of the original polymer, were preceded and followed by purine nucleotides.

ACKNOWLEDGEMENTS

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STUDIES ON THE NUCLEOTIDE ARRANGEMENT IN DEOXYRIBONUCLEIC ACIDS

IV. PATTERNS OF NUCLEOTIDE SEQUENCE IN THE DEOXYRIBONUCLEIC ACID OF RYE GERM AND ITS FRACTIONS

HERMAN S. SHAPIRO AND ERWIN CHARGAFF

Cell Chemistry Laboratory, Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y. (U.S.A.)

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SUMMARY

The procedure of the differential analysis of pyrimidine nucleotide distribution has been applied to the total deoxyribonucleic acid of rye germ and to a complete series of seven DNA fractions prepared by the fractional dissociation of rye germ nucleoprotein. The fractions exhibited the previously observed gradation in composition with gradually rising adenine and thymine contents and similarly diminishing concentrations of guanine, cytosine and methylcytosine.

The general distribution of cytosine and methylcytosine in the fractions was definitely uneven, with a relatively higher proportion of the methyl derivative occurring in the first fractions. Even greater differences were observed in the relative distribution of solitary pyrimidine nucleotides, that of thymidylic and methylcytidylic acids showing a remarkable similarity and exceeding by far the corresponding contribution of cytidylic acid to the solitary units.

The survey of solitary units was supplemented by a study of the distribution of many of the pyrimidine dinucleotide sequences that are flanked by purine nucleotides in the polymer chains. Significant differences in the composition of the fractions were observed, but in all preparations more than two thirds of the pyrimidines occurred as tracts consisting of three or more pyrimidines in a row. In no case was the pattern of nucleotide distribution that to be expected from a random arrangement of constituents.

After the discussion of certain outstanding features of the nucleotide arrangement, including the evidence for a preferential association of methylcytidylic and guanylic acids, the bearing of the findings on schemes of DNA replication is considered.

INTRODUCTION

The differential distribution analysis of pyrimidine nucleotides in deoxyribonucleic acids developed by us a few years ago[1-3] provides a method for the characterization of specific structural differences that goes far beyond the information obtainable from constituent analysis. Owing to the great lability to acid of the glycosidic bond of the purine nucleosides, which is in contrast to the relative stability of the pyrimidine deoxyribosides, the purines can be stripped from the polymer under proper conditions, giving rise to an apurinic acid in which the pyrimidine nucleotides remain aligned as in the parent nucleic acid, whereas reactive aldehydo groups now mark the positions originally occupied by the purines[4]. If a somewhat more vigorous acid treatment is applied to the nucleic acid, the degradation goes beyond the stage of apurinic acid and leads, through a series of phosphate eliminations at the sites of the interjacent purine nucleotides, to the detachment of all pyrimidine nucleotide tracts, as they existed in the original polymer, carrying terminal phosphate groups on both ends[2]. The shortest members of this series are the pyrimidine nucleoside 3′,5′-diphosphates. They are released in the first stage of the degradative procedure to an extent varying with, and characteristic of, their sequential position in the polymer chain, so long as they occur in the latter as what we have called solitary units, namely, as a single pyrimidine flanked on both sides by purine[3]. At the same time "bunched" pyrimidine oligonucleotides of varying length and composition are liberated, again indicative of the sequence characteristics of the parent chain. These can be further and distinctively degraded in later stages of acid treatment as has been discussed before[2,3].

One of the uses to which the differential analysis procedure may be put is the demonstration that deoxyribonucleic acids from different sources, but showing no differences in the proportions of their nitrogenous constituents[5,6], exhibit entirely different sequence characteristics[3]. Other conclusions from our previous work will be discussed below in the light of the present studies. These deal with the deoxyribonucleic acid of rye (*Secale cereale*) which contains 5-methylcytosine as a minor, though considerable, component. Both the total preparation and a complete series of fractions were examined. The present work affords, for the first time, an opportunity of studying the structural characteristics of all nucleic acid fractions obtained by the fractional dissociation of a nucleoprotein[5] and makes it possible, through a comparison of these findings with those recorded for a total nucleic acid preparation, to gain a measure of the validity of the procedures. Moreover, the presence in these nucleic acids of appreciable quantities of a pyrimidine that shares with cytosine the property of being a 6-amino derivative, and with thymine that of being a 5-methyl compound, invites a sharper definition both of the pattern in which these pyrimidines appear in, or are selected for incorporation into, the polymer chain and of the manner in which they replace each other. The findings presented here may be of immediate use in setting limits to certain replication mechanisms; but the ultimate aim of this or any method for the elucidation of nucleotide sequence is that of gaining some understanding of the relationship between structure and function.

EXPERIMENTAL[*]

Preparation of DNA

Unprocessed rye germ, which was obtained through the courtesy of F. H. BLODGETT, Inc., Janesville, Wisconsin, and King Midas Flour Mills, Minneapolis, Minnesota, was separated from contaminating endosperm and bran fragments by a flotation procedure with the use of a mixture of cyclohexane and chloroform[8]. After being defatted further by extraction in the cold with petroleum ether overnight, 210 g of the air-dried material were ground in a mortar. The isolation of DNA and its analysis followed, with a few modifications, the previously described procedures for wheat germ DNA[9]. The DNA preparation weighed 1.18 g and was free of ribonucleic acid. Its composition is presented in Table I which includes, for purposes of comparison, our previous analysis of wheat germ DNA. It will be seen that the DNA preparations from both cereal species are indistinguishable with regard to nitrogenous base contents. We have been unable to confirm the unusually high proportion of 5-methylcytosine (10.0 mole %) in rye DNA that has been claimed in the literature[10].

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[*] The presentation of structures has been described in the preceding paper[7]. In addition to the abbreviations defined there, A will designate deoxyadenosine, G deoxyguanosine, Pu purine deoxyriboside.

TABLE I

COMPOSITION OF DEOXYRIBONUCLEIC ACIDS OF RYE GERM AND WHEAT GERM

	*Rye germ**	*Wheat germ***
	Moles/100 g-atoms P	
Adenine	27.7	28.1
Guanine	22.6	21.8
Cytosine	16.4	16.8
Methylcytosine	5.9	5.9
Thymine	27.4	27.4
	Molar ratios	
Adenine + thymine to guanine + cytosine + methylcytosine	1.23	1.25
Purines to pyrimidines	1.01	1.0
Adenine to thymine	1.01	1.03
Guanine to cytosine + methylcytosine	1.01	0.96
Cytosine to methylcytosine	2.78	2.85

* The figures have been corrected for 100% recovery; the actual recovery was 101%.
** Taken from a previous publication[9].

Fractionation of DNA

The deoxyribonucleoprotein of rye germ was isolated by the procedure that had served for the preparation of the analogous material from wheat germ[9]. A solution of the nucleoprotein containing a total of 135 mg of DNA phosphorus was subjected to fractional dissociation in the presence of chloroform, as described before[9], over the range of 0.6 to 1.7 *M* NaCl. Seven fractions were collected accounting for 87 % of the starting DNA (in terms of P). Purification and analytical techniques followed our previous procedures. The composition of the fractions is shown in Table II. When the increments contributed by each fraction were computed, correction being made for the total recovery of 87 % of the DNA phosphorus, a reconstructed composition of the total DNA could be established which is included in Table V to be discussed later. The comparison with the actual findings in Table I shows excellent agreement and demonstrates, moreover, that no significant fraction had been lost.

Stepwise hydrolysis of DNA

The acid degradation of the DNA preparations was performed in the hydrolysis

TABLE II

COMPOSITION OF RYE GERM DNA FRACTIONS PREPARED BY FRACTIONAL DISSOCIATION OF NUCLEOPROTEIN GELS

No.	*Fraction*		*Moles/100 g-atoms P**					*Molar ratios*				
	NaCl molarity	*% of DNA in starting material*	*A*	*G*	*C*	*M*	*T*	$\frac{A+T}{G+C+M}$	$\frac{Pu}{Py}$	$\frac{A}{T}$	$\frac{G}{C+M}$	$\frac{C}{M}$
I	0.60	12.9	25.9	23.9	17.0	6.8	26.3	1.09	0.99	0.98	1.00	2.50
II	0.65	11.2	27.4	22.9	16.2	6.4	27.1	1.20	1.01	1.01	1.01	2.53
III	0.70	17.8	27.5	21.6	16.6	6.1	28.2	1.26	0.96	0.98	0.95	2.72
IV	0.75	14.4	28.1	21.8	16.6	5.8	27.8	1.26	0.99	1.01	0.97	2.86
V	0.80	9.2	27.9	21.2	16.8	6.1	28.0	1.27	0.96	1.00	0.93	2.75
VI	0.90	16.2	28.9	20.7	16.5	5.4	28.6	1.35	0.98	1.01	0.95	3.06
VII	1.7	5.3	30.3	20.0	15.8	4.7	29.2	1.47	1.01	1.04	0.98	3.36

* The abbreviations refer to the nitrogenous constituents. The mean proportions of each constituent have been corrected for a 100% recovery.

arrangement described before[2,3]. Suspensions of the DNA fibers (2% by weight in 0.1 M H_2SO_4) were prepared directly in the hydrolysis tube and heated at 100° under reflux, 50 to 60 mg of DNA being used in each experimental series. Portions of the hydrolysates were removed after 30 min (Stage I), 60 min (Stage II) and 120 min (Stage III) and diluted to an exact volume of 100 ml with 0.01 M aqueous ammonia. After the removal of 1-ml portions for the estimation of total and inorganic P, the fluids were applied to Dowex-2-formate columns.

Analysis of hydrolysates

As was shown in the preceding communication (Fig. 1)[7], with a suitable gradient concentration and with slow gradient changes, the order of elution of the pertinent components is pMp, pCp, pMpCp*, pCpCp, pTp, pMpTp* and pCpTp*. For routine analysis, however, it was found more convenient to adjust the changes in buffer concentration somewhat more abruptly so that the elution pattern comprised only four peaks, namely, (I) pMp + pCp; (II) pMpCp + pCpCp; (III) pTp; and (IV) pMpTp + pCpTp (see Fig. 1). A known quantity of thymidylic acid (0.5 µmole) was

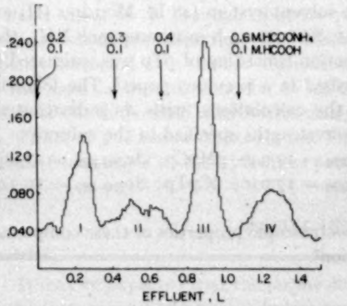

Fig. 1. Concentration gradient elution pattern of Stage I acid hydrolysate of rye germ DNA fraction IV. Conditions: Volume of mixing chamber, 250 ml; the arrows underneath the indicated formate buffer concentrations (upper figure ammonium formate, lower figure formic acid) denote points of change in the contents of the single reservoir; initial buffer strength in the mixing chamber was 0.1 M $HCOONH_4$–0.1 M HCOOH; Dowex-2 (8 ×), 100–200 mesh, 15 cm × 1.3 cm diam.; 10-ml fractions.

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* These components presumably comprised both sequential isomers.

TABLE

RATES OF RELEASE OF PYRIMIDINE NUCLEOSID

Preparation	*Total DNA*	*Fraction I*	*Fraction II*
Moles T/100 g-atoms P	27.4	26.3	27.1
Moles C/100 g-atoms P	16.4	17.0	16.2
Moles M/100 g-atoms P	5.9	6.8	6.4
T/C, molar ratio	1.67	1.55	1.67
C/M, molar ratio	2.78	2.50	2.53
T/M, molar ratio	4.64	3.87	4.23

STEPWIS

Stage	*I*	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*
pTp, as mole % T	17.8	20.8	22.5	14.4	18.7	20.3	14.4	15.6	18.1
Average deviation	1.9	0.8	0.7	1.3	0.8	0.6	1.8	0.5	3.0
pCp, as mole % C	11.0	11.0	9.5	7.4	8.5	7.5	10.6	10.4	10.2
Average deviation	—	—	—	0.5	0.9	1.3	1.5	1.9	3.9
pMp, as mole % M	17.8	18.4	18.1	12.3	12.7	12.8	18.2	18.6	18.2
Average deviation	—	—	—	0.3	2.4	2.4	2.3	4.0	6.9
pTp, as mole % P	4.89	5.71	6.16	3.79	4.92	5.34	3.89	4.22	4.90
pCp, as mole % P	1.81	1.81	1.55	1.26	1.45	1.28	1.72	1.68	1.65
pMp, as mole % P	1.05	1.09	1.07	0.84	0.86	0.87	1.16	1.19	1.16
pTp/pCp, molar ratio	2.70	3.15	3.97	3.01	3.39	4.17	2.26	2.51	2.97
pCp/pMp, molar ratio	1.72	1.67	1.45	1.50	1.69	1.47	1.48	1.41	1.42
pTp/pMp, molar ratio	4.66	5.24	5.76	4.51	5.72	6.14	3.35	3.55	4.22

[*] The mole % figures refer to moles/100 moles of indicated constituent present in DNA.

added to each of the three mixed fractions as an internal standard which served for the estimation of the yields of the respective substances after their separation by paper chromatography. For this purpose, the eluates were neutralized with aqueous ammonia, lyophilized and freed of ammonium formate by sublimation. Portions of the solutions of the residues were chromatographed on Whatman paper No. 1. Mixture (I) was separated by means of the isobutyrate solvent system (48 h). Mixtures (II) and (IV) required two-dimensional development: first for 24 h in isopropanol–NH_3, then for 48 h in the isobutyrate system. The fraction consisting of pTp was estimated by direct spectroscopy of the eluate, as described in a previous paper[3]. The following molar extinction values[3,7] were used for the calculations, with $\Delta\varepsilon$ indicating the difference in the ε values recorded at the wavelengths specified in the subscript.

pMp: $\Delta\varepsilon_{290-320} = 12{,}000$; pCp: $\Delta\varepsilon_{280-320} = 12{,}940$; pMpCp: $\Delta\varepsilon_{280-320} = 21{,}660$; pCpCp: $\Delta\varepsilon_{280-320} = 23{,}790$; pMpTp: $\Delta\varepsilon_{280-320} = 17{,}610$; pCpTp: $\Delta\varepsilon_{280-320} = 19{,}320$; pTp: $\Delta\varepsilon_{264-320} = 8{,}960$.

The chromatographic mobilities and spectroscopic properties of these compounds can be found in the preceding communication[7].

Presentation of analytical results

The correction factors for the loss of the diphosphates pTp and pCp taking place

II

IPHOSPHATES FROM RYE GERM DNA AND FRACTIONS*

Fraction III	*Fraction IV*	*Fraction V*	*Fraction VI*	*Fraction VII*
28.2	27.8	28.0	28.6	29.2
16.6	16.6	16.8	16.5	15.8
6.1	5.8	6.1	5.4	4.7
1.70	1.67	1.67	1.73	1.85
2.72	2.86	2.75	3.06	3.36
4.62	4.79	4.59	5.30	6.21

YDROLYSIS**

I	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*
16.8	18.7	20.5	16.6	19.0	22.0	14.1	17.5	21.4	18.8	22.2	23.9	15.2	17.7	18.0
1.2	1.2	0.8	1.3	0.6	0.6	1.6	1.2	0.7	0.7	1.4	2.3	0.8	2.1	4.1
9.7	10.0	10.0	11.8	10.8	12.3	8.8	8.2	8.4	12.6	11.4	9.9	9.8	11.5	12.0
1.9	1.4	0.7	1.2	2.0	1.4	1.5	0.3	0.9	0.5	0.8	1.1	0.4	2.5	2.6
16.6	15.1	18.3	20.1	19.6	22.6	15.9	14.8	16.5	22.0	22.9	21.8	18.0	21.0	21.8
3.5	0.7	1.6	0.9	2.9	1.5	2.6	1.0	0.5	—	0.2	2.1	0.6	2.2	2.3
4.73	5.26	5.77	4.61	5.28	6.12	3.95	4.90	5.99	5.38	6.35	6.84	4.44	5.17	5.26
1.61	1.66	1.66	1.96	1.79	2.04	1.48	1.38	1.41	2.08	1.88	1.63	1.55	1.82	1.90
1.01	0.92	1.12	1.17	1.14	1.31	0.97	0.90	1.01	1.19	1.24	1.18	0.85	0.99	1.02
2.94	3.17	3.48	2.35	2.95	3.00	2.67	3.55	4.25	2.59	3.38	4.20	2.86	2.84	2.77
1.59	1.80	1.48	1.68	1.57	1.56	1.53	1.53	1.40	1.75	1.52	1.38	1.82	1.84	1.86
4.68	5.72	5.15	3.94	4.63	4.67	4.07	5.44	5.93	4.52	5.12	5.80	5.22	5.22	5.16

** The values for nucleoside diphosphates liberated during the hydrolysis stages I, II, III (30, 60, 120 min, respectively, in 0.1 *M* H_2SO_4 at 100°) have been corrected for the decomposition of the diphosphates occurring in the course of this treatment. Each figure is based on the results of at least two independent hydrolysis experiments. The spread between the means recorded in each hydrolysis is indicated by the average deviation.

by destruction after their liberation from precursor sources during the acid hydrolysis of DNA and examples of the use of these factors have been reported previously[8]. As the *k* value for pCp equals that for pMp[7], the same correction factor could be applied to both diphosphates. The yields of the dinucleoside triphosphates were similarly corrected with the use of the factor for pCp (pMp), since these diphosphates are the most labile unit common to all these products. Table III summarizes the results of the differential distribution analysis of all preparations with regard to the production of nucleoside diphosphates. The production of dinucleoside triphosphate fragments at stage I of the hydrolysis procedure is recorded in Table IV.

RESULTS AND DISCUSSION

Material

It may be expected that the purine and pyrimidine satellites, *i.e.* the nitrogenous constituents present in a relatively low concentration in nucleic acids, will assume an increasing importance for the advance in our understanding of the structure, the

TABLE IV

RELEASE OF DINUCLEOSIDE TRIPHOSPHATES FROM DNA FRACTIONS AT HYDROLYSIS STAGE I

Fraction No.	*pMpCp**			*pCpCp*		*Molar ratio*	*pMpTp**			*pCpTp**			*Molar ratio*
	*Mole % DNA-P***	*Pyrimidine content****		*Mole % DNA-P***	*Cytosine content****	$\frac{pCpCp}{pMpCp}$	*Mole % DNA-P***	*Pyrimidine content****		*Mole % DNA-P***	*Pyrimidine content****		$\frac{pCpTp}{pMpTp}$
		Methyl-cytosine	*Cytosine*					*Methyl-cytosine*	*Thymine*		*Cytosine*	*Thymine*	
I	0.22	3.2	1.3	0.30	3.5	1.4	0.45	6.6	1.7	0.79	4.6	3.0	1.8
II	0.27	4.2	1.7	0.40	4.9	1.5	0.52	8.1	1.9	1.12	6.9	4.1	2.2
III	0.25	4.1	1.5	0.35	4.2	1.4	0.53	8.7	1.9	1.24	7.5	4.4	2.3
IV	0.25	4.3	1.5	0.35	4.2	1.4	0.48	8.3	1.7	1.09	6.6	3.9	2.3
V							0.43	7.0	1.5	1.06	6.3	3.8	2.5
VI	0.18	3.3	1.1	0.30	3.6	1.7	0.39	7.2	1.4	0.89	5.4	3.1	2.3
VII	0.23	4.9	1.5	0.45	5.7	2.0	0.38	8.1	1.3	0.80	5.1	2.7	2.1

* This component presumably comprised both sequential isomers.

** As moles of dinucleoside triphosphate/100 g-atoms of DNA phosphorus.

*** As moles/100 moles of the particular pyrimidine in the DNA fraction. The composition of the fractions is shown in Table II.

nucleotide sequence and the mode of biosynthesis of the nucleic acids. For this reason, a deoxyribonucleic acid of plant origin was chosen for the present study, as these substances are known to contain a considerable proportion of a third pyrimidine, *viz.*, 5-methylcytosine[11]. A glance at Table I will show that the deoxyribonucleic acid of rye germ under investigation here is, in its composition, indistinguishable from the analogous preparation from wheat germ studied previously[9]. This similarity is perhaps not surprising, since the two species are closely related biologically, wheat being capable of crossing with rye[12,13]. Even more noteworthy is the resemblance of the rye germ nucleic acid fractions, prepared by the method of fractional dissociation of the nucleoprotein[14], to those similarly isolated from wheat germ preparations[9] (see Table II). This far-reaching similarity between the two cereal species will make it possible subsequently to correlate the findings discussed here with some of the results of the work on wheat germ DNA.

The rye germ DNA fractions listed in Table II exhibit the principles of composition stressed before[14]: they represent a graded series of polymers with increasing contents of adenine and thymine and diminishing contents of guanine and cytosine + methylcytosine. As in all previous cases studied in this laboratory the various complementariness relationships[5] were all maintained; it will be seen that the ratios of adenine to thymine, guanine to cytosine + methylcytosine, purines to pyrimidines, and 6-amino to 6-keto nucleotides all were near 1.

Procedures

The various procedures for the fractionation of deoxyribonucleic acids have been discussed repeatedly[5,9,15-17]. They may vary in practicability or even trustworthiness, but have in common that their rationale, let alone their import, is far from understood. There can, however, be little doubt that the first fractionation procedure to be described[14], which is the one used here, permits the isolation, in quantity and in good yield, of a series of fractions, each of which possibly represents a cluster of closely related polymers. Fractionation arrangements, on the other hand, that prescribe conditions normally shunned during the preparation of nucleic acid can hardly be accepted as representing a true segregation of members of a nucleic acid population. For instance, a recently described procedure[18] which employs fractional precipitation with mineral acid must lead to conglomerates of degradation products rather than to fractions. Deoxyribonucleic acid subjected to a mildly acidic environment is known to suffer a measurable loss of purine and degradation of its polymer structure[19].

As regards the studies on the distribution of pyrimidine nucleotides (Table III), the analytical results obtained at the three hydrolysis stages are essentially indicative of two distinct structural features of the nucleic acid polymer. The more easily interpreted data on the nucleoside diphosphates and dinucleoside triphosphates released on short exposure to acid, *i.e.* in Stage I, furnish a direct indication of the number of such units—"solitary" p Py p and "bunched" p Py p Py p[3]—that in the nucleic acid chain were flanked by purine nucleotides.

The increase in the production of these compounds during more prolonged acid hydrolysis (Stages II and III) reflects their secondary slow release from larger polypyrimidine nucleotide units. The slope of p Py p production between Stages I and III, although more difficult to interpret, may indicate the relative abundance of "bunched" units of a certain length and composition in different nucleic acid preparations[3]. The inspection of Table III will demonstrate that between Stages I and III there occurs, in general, an increase of about 30 % of pTp over the 30-min value, a 7 % rise of pMp and practically no increase in pCp: obviously a reflection of the decay of available pyrimidine oligonucleotide tracts during this interval. As concerns the smallest "bunched" units, namely, those emerging as dinucleoside triphosphates in Stage I (Table IV), it should be mentioned that from our observations their production (after proper correction) shows only a slow increase between Stages I and III, in accordance with our previous findings with one of these oligonucleotides[3].

Liberation of inorganic phosphate

Although some use has been made of the rate of liberation of inorganic phosphate for structural interpretation[20,21], it is difficult to correlate these data with features of the purine nucleotide arrangement in deoxyribonucleic acids. In our present work, the total nucleic acid and its fractions exhibited identical rates of liberation of inorganic phosphate. In all cases, 15 % of the DNA phosphorus were released as inorganic phosphate in Stage I, 22 % in Stage II, 30% in Stage III. This phosphate must have originated from phosphate bridges between purine nucleotides. We believe that under our experimental conditions this release—the doubling of the inorganic phosphate between 30 and 120 min of hydrolysis—is independent of the more immediate liberation of pyrimidine units by a series of elimination reactions and occurs at a relatively steady rate.

Test of the validity of the findings

As we pointed out in the beginning, this is the first time that a complete series of fractions, in addition to the preparation of total deoxyribonucleic acid, was examined for finer features of nucleotide arrangement. This afforded an opportunity to test the validity of the results obtained with the individual fractions by reconstructing a weighted average that could be compared with the values recorded for the total DNA preparation which had not undergone fractionation. The compilation in Table V shows a truly excellent agreement in most instances.

General distribution of methylcytosine in DNA fractions

We had noticed previously[5,9,14,22]—and discussed these observations in some detail[23]—that the trend of the distribution of 5-methylcytosine in DNA fractions from calf thymus and wheat germ differed from that of the abundance of cytosine; in other words, it was impossible to claim that these two 6-amino pyrimidines replaced each other at random within the polynucleotide chain. The same observation was made in the present study of deoxyribonucleic acid fractions of rye germ. As will be gathered from Tables II and VI, the distribution of methylcytosine with respect to cytosine is uneven, the percentage contribution of the minor pyrimidine being higher in the earlier fractions. We shall return to this point later, but wish to point out that the tendency observed here very much resembles our previous findings with wheat germ DNA[9].

"Solitary" pyrimidine nucleotides and evidence of specific nucleotide sequences

The following discussion will limit itself to Stage I of the differential hydrolysis procedure: the diagnostically most significant interval, since it permits a decision

TABLE V

COMPARISON OF FOUND AND RECONSTRUCTED COMPOSITION OF TOTAL RYE GERM DNA*

		Found	*Reconstructed*
Nitrogenous constituents	Adenine, moles/100 g-atoms P	27.7	27.8
	Guanine, moles/100 g-atoms P	22.6	21.8
	Cytosine, moles/100 g-atoms P	16.4	16.6
	Methylcytosine, moles/100 g-atoms P	5.9	6.0
	Thymine, moles/100 g-atoms P	27.4	27.8
	Methylcytosine, moles/100 moles of methylcytosine + cytosine	26.5	26.5
Solitary pyrimidine nucleotides	pMp, moles/100 moles of methylcytosine in DNA	17.8	17.8
	pCp, moles/100 moles of cytosine in DNA	11.0	10.3
	pTp, moles/100 moles of thymine in DNA	17.8	16.1

* Comparison and computation are based on the data in Tables I, II, III and VI. The reconstructed values represent weighted balances established from the findings on Fractions I–VII. The figures for solitary pyrimidine nucleotides are computed from the results obtained in Stage I (Table III).

TABLE VI

UNEVEN DISTRIBUTION OF 5-METHYLCYTOSINE IN RYE GERM DNA FRACTIONS*

Methylcytosine as mole % of cytosine + methylcytosine in fractions						
I	*II*	*III*	*IV*	*V*	*VI*	*VII*
28.6	28.3	26.9	25.9	26.6	24.7	22.9

* The fractions are numbered as in Tables II to IV.

on the number of pyrimidine mononucleotides that are contiguous only to purine nucleotides within the high polymer chain. Surveying the results, given in Table III, as a whole, we notice first that a surprisingly large proportion of methylcytosine, and a surprisingly small proportion of cytosine, occur as solitary units. The variation in the percentages of solitary units present in the various fractions (as mole % of the particular pyrimidine) covers the following range: methylcytosine, 12.3 to 22.0; cytosine, 7.4 to 12.6; thymine, 14.1 to 18.8. These unexpected findings give added emphasis to what has been said in the preceding section about the inability of the two 6-amino pyrimidines to take each other's place in an automatic fashion and they indicate at once that methylcytidylic acid and cytidylic acid do not share a common pattern of nucleotide sequence.

If the occurrence of a trinucleotide sequence comprising pCp is expressed as a probability factor, then—since total purine equals total pyrimidine[5]—the probability of pC appearing in a Pu pCp Pu arrangement would be $[0.5 \times pC/P \times 0.5]$ or 0.25 of the total cytidylic acid, with 0.5 and pC/P referring to the respective frequencies of purine nucleotides and cytidylic acid per g-atom of nucleic acid phosphorus[3]. The same probability relation, one quarter, should apply to pM or to pT appearing as solitary units. This relationship holds in no case experimentally (Table III); and this fact, pointed out before, was among the bases of our conclusion, reaffirmed now, that the nucleotide arrangement in all deoxyribonucleic acids is far from random[3].

The experiments summarized in Table III demonstrate, in addition, that, if the relative contributions of the three pyrimidines to the sequence Pu p Py p Pu are compared, that of methylcytosine is on the average 70 % higher than that of cytosine; a value that appears to be quite constant among the fractions. For instance, in Fraction I (isolated by means of 0.6 *M* NaCl) 12.3 % of the total pM appears as the solitary nucleoside phosphate as contrasted with 7.4 % of the total pC: a ratio of 1.66:1; in Fraction VI (0.9 *M* NaCl) the corresponding ratio is 1.75, in Fraction VII (1.7 *M* NaCl) 1.84. These results—and they apply to all fractions as well as to the total DNA preparation—disclose that the probability of methylcytosine being flanked on both sides by purines is nearly twice that of cytosine being so enclosed. We shall, in fact, later mention the evidence that the sequence pMpG occurs in this nucleic acid in a uniquely high frequency.

On the other hand, in a similar comparison, again based on Table III, the mole fractions of pM and pT appearing as solitary units are nearly equal; a point to which we shall return below in another context.

We now turn to the consideration of "bunched" sequences. In the course of analyzing the mixture of the dinucleoside triphosphates pMpCp and pCpCp (compare Fig. 1) from a hydrolysate of Fraction IV (Table IV) a faint u.v.-absorbing zone with a mobility slightly greater than that of pMpCp was noticed after two-dimensional paper chromatography. The absorbance ratio A_{280}/A_{260} in 0.01 *N* HCl was 2.6. This component was undoubtedly pMpMp, as hydrolysis with concentrated formic acid produced only methylcytosine and inorganic phosphate. The yield of pMpMp was estimated as 0.068 mole/100 g-atoms of DNA phosphorus, equivalent to 2.3 % of the DNA methylcytosine*. This is another instance of the dissimilarity in the manners of insertion of methylcytosine and cytosine in the DNA chain: in the same preparation, Fraction IV, 4.2 mole % of cytosine are found in the analogous pCpCp unit (Table IV). On the other hand, the positional resemblance of thymine and methylcytosine is again indicated by the data in Table IV. Approximately 4 mole % of the methylcytosine are found in the form of pMpCp and a similar molar proportion of thymine in the analogous component pTpCp.

Additional information on the trends of association of methylcytidylic acid with other nucleotides can be gathered through the comparison of the molar quantities of "bunched" units listed in Table IV. For the ratio of pCpCp to pMpCp the values range between 1.4 and 2.0, for that of pCpTp to pMpTp between 1.8 and 2.5. In all instances these figures are lower than the corresponding ratios of total cytosine to methylcytosine in the several fractions which, as pointed out before, show a regular gradation from 2.5 to 3.4. One may infer that—at least with regard to dipyrimidine units—the arrangement of pM and pC is such as to make for a greater tendency of pM to be linked to either pT or pC than of pC to associate with itself or with pT; and this with a relative bias in favor of pMpCp.

One other conclusion can be drawn from the information in Tables III and IV.

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* The corresponding dinucleoside triphosphate pTpTp was not isolated in the course of these studies; but it probably accounts for a similar percentage of the total thymine of the DNA[3].

Methylcytosine makes, proportionally, the greatest contribution to the sum of solitary and dipyrimidine nucleotide units, up to 35 % (in Fraction IV) of the total methylcytosine being so accounted for; the highest corresponding figure for both thymine and cytosine is 23 to 24 %. This means that more than two thirds of the pyrimidines of the deoxyribonucleic acid—and therefore also of the purines—are engaged in associations comprising at least three of one kind in a row[3]. It may be expected that much will be learned about the specificity patterns of nucleic acids when methods for the separation and characterization of these larger oligonucleotide fragments become available.

Family resemblance of nucleic acid fractions and general features of nucleotide arrangement

Two impressive points of resemblance, suggesting a limited similarity in the patterns of nucleotide arrangement in a series of deoxyribonucleic acid fractions derived from the same cellular source, have been mentioned in the preceding section; there is an approximately constant relationship between the mole fractions of the total individual pyrimidines appearing as solitary units. In general, a comparison of the results summarized in Table III with some of our previous findings on nucleic acids from other sources[3] would lead to the conclusion that there is a greater similarity between differently composed fractions of identical origin than between preparations of identical total composition but derived from different organisms (see the comparison in Table I of a previous paper[1]). In other respects, the various fractions differ considerably; for instance, to take two extreme cases, 11.8 % of the pyrimidines of Fraction I appear as solitary units, but 17.3 % of the pyrimidines of Fraction VI. It is, therefore, not possible to postulate the existence of a polynucleotide framework that would be common to all DNA fractions of a given nucleus.

The criteria underlying the decision whether the distribution of nucleotides in deoxyribonucleic acid is consistent with a randomly arranged sequence of units have already been discussed in detail[3]. In a mixed linear polymer constructed at random the solitary pyrimidine units and the bunched dipyrimidine units should each account for 25 % of the pyrimidine content of the nucleic acid; and the same expectation should hold for the purines. In all instances discussed in the preceding paragraphs the experimentally found values are far below those predicted for a random arrangement; and we can say that our present findings on a plant deoxyribonucleic acid substantiate those previously reported by us for preparations of animal origin[3]: in all nucleic acids that have been subjected to differential distribution analysis the sequential arrangement of constituents appears to be far from random.

It may be remarked that another corollary of randomness also is rarely encountered experimentally. The molar ratios of the individual solitary pyrimidine units and of the dipyrimidine units sharing one of these pyrimidines should equal the corresponding interpyrimidine ratios of the nucleic acid. For instance, the relationship pCp/pMp = pCpCp/pMpCp = total C/total M holds for no case examined here. Only in two instances, the total DNA and Fraction III, are the expressions pTp/pMp and total T/total M numerically identical; but even in Fraction III, the one case for which all data are available, this equality does not extend to the ratio pCpTp/pMpCp.

Bearing of present findings on specific selection mechanisms

The nonrandomness of nucleotide sequence in a deoxyribonucleic acid may be taken to signify that molecular replication or, more specifically, the insertion of a particular nucleotide at a particular place of the forming polymer chain occurs according to a plan. Once this premise is accepted, a search for guiding principles may be relevant: patterns that could reveal to us what kind of plan we are dealing with. It could, of course, be that there are no principles or that they are not obvious enough for us to grasp.

The case in hand presents the following situation. We are concerned with a total DNA preparation from rye germ and with a complete series of DNA fractions all of which exhibit the well known unity relationships[5]. These include—it is important to remember—the equality of the molar sums of 6-keto nucleotides and 6-amino nucleotides as well as the presence of equal molar quantities of the 6-ketopurine guanine and of the sum of the two 6-aminopyrimidines cytosine and 5-methylcytosine (compare Table II). The latter compound, moreover, occurs in wheat germ DNA and in rye germ DNA in identical molar concentrations (compare Table I). The first observation, the equality of G and M + C, could have led to the conclusion that these two pyrimidines replace each other at random in the polynucleotide chain, selection having regard only to the presence of a 6-amino group in the pyrimidine; but the second observation, the surprisingly constant molar quantity of methylcytosine so incorporated, should have served to throw doubt on this conclusion. Even more surprising was the finding, repeated in the present study, that when DNA was separated into a series of regularly graded fractions a disproportionate distribution of cytosine and methylcytosine was observed, the latter being relatively higher in the early fractions[9,14,22]. The bearing of these results on current replication hypotheses was discussed by one of us some time ago[23].

The present investigation goes further. Making use of the concepts of solitary and bunched nucleotides introduced by us previously[3], it supplies information on the distribution density of each of the three pyrimidines as it occurs, as a solitary nucleotide or as a pyrimidine dinucleotide, flanked by purines. The results were entirely unexpected; there is no evidence that the two 6-aminopyrimidines are treated indiscriminately by the selection mechanism, but some indication that no distinction is exercised in regard to the two 5-methylpyrimidines. The results bearing on this point have been discussed in the preceding sections of this communication; but we should like to add one more observation. Although, as pointed out above, the total number of solitary pyrimidines is not constant in all fractions of the same DNA and varies over a wide range in DNA preparations from different organisms[3], the results of the distribution studies in Table III can be compared easily in form of the amounts of individual solitary pyrimidine per 100 solitary places occupied by pyrimidines in the given preparation. For instance, in the total DNA preparation of rye germ we find 63 % thymidylic acid, 23 % cytidylic acid and 14 % methylcytidylic acid as solitary units; this means that 77 % of the solitary spaces are filled by 5-methylpyrimidines (T + M) and 37 % by 6-aminopyrimidines (C + M). If the last two expressions are computed for all seven fractions, there becomes noticeable a much wider spread in the percentages of total 6-aminopyrimidines than of total 5-methylpyrimidines. The mean percentage of solitary thymine + methylcytosine is 76.4 ± 1.4, with a coefficient of variation of only 1.8; that of solitary cytosine + methylcytosine is 37.9 ± 2.6, with a coefficient of variation of 6.8.

A copying mechanism that limits itself to a specific pairing between a 6-amino-purine and a 6-ketopyrimidine or between a 6-ketopurine and a 6-aminopyrimidine[24] must, consequently, be incorrect or so incomplete as to become meaningless. It may, however, be regretted that it is necessary to replace a handsome, though unfounded, hypothesis by an unpleasant, though demonstrable, paradox. This is the paradox: on the one hand, if the total composition of a deoxyribonucleic acid is considered, the molar proportions of the 6-ketopurines equal those of the 6-aminopyrimidines; but on the other hand, if the individual positions of these pyrimidines within the polymer chain are examined, a discrimination in their selection becomes noticeable. One gains the impression that two selection mechanisms are superimposed on each other; one control being exercised by the matrix in which the newly forming polynucleotide chain is made, expressing itself through the complementariness principles mentioned before; the other consisting in a restriction imposed by the neighboring nucleotides of the forming polymer itself. This possibility was mentioned by one of us some time ago[23].

Our results have shown that cytosine and its methyl derivative do not exchange positions haphazardly. If, however, the insertion of a structural unit depended on the identity of the adjacent nucleotides, it could be understood that of the sequences pCpG, pCpA, pCpT or pCpC perhaps only one may be capable of accepting methylcytidylic instead of cytidylic acid. The change from 2.5 to 3.4 in the ratio of total cytosine to total methylcytosine contained in the several fractions (Table II) could, for instance, be explained by assuming a scheme involving the random substitution of pCpG by pMpG*. The number of pCpG sequences present in a deoxyribonucleic acid cannot yet be determined and it presumably varies from one polymer to another in relation to the other sequences comprising pC; but the probability of such a postulate can be evaluated tentatively from the figures in Table II. In the seven fractions under examination, the ratio of guanine + cytosine to methylcytosine is indeed much more constant (coefficient of variation 2.5) than the ratio of cytosine to methylcytosine or of adenine + cytosine to methylcytosine which show coefficients of variation of 10 and 14, respectively.

Also in other respects the suggestion that guanylic acid may influence the disposition of methylcytidylic acid is not entirely arbitrary. The analysis of the dinucleotides produced from both calf thymus and wheat germ DNA by pancreatic deoxyribonuclease has revealed that an extraordinarily large proportion of the methylcytidylic acid is esterified with guanylic acid[25, 26]. As produced from calf thymus DNA, this dinucleotide has been shown to be all pMpG[27]. These observations are very significant, despite some uncertainty concerning the mode of enzymic action.

Although the analysis of the distribution of solitary pyrimidine nucleotides in wheat germ DNA has revealed a peculiar equivalence of the two 5-methylpyrimidines thymine and methylcytosine, it cannot yet be decided whether the main emphasis should be given to the presence of the methyl substituent or the absence of a hydrogen atom in position 5 of the pyrimidine. We are still very far from an understanding of the stereochemistry of nucleic acid replication.

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* It is, of course, understood that not all of the methylcytidylic acid of the DNA is next to guanylic acid. We have described here the isolation of pMpMp; and evidence for the occurrence of the sequence ApMpTp in an enzyme digest of wheat germ DNA has also been reported[25].

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A METHOD FOR THE SYNTHESIS OF RIBONUCLEOSIDE AND DEOXYRIBONUCLEOSIDE-5′-TRIPHOSPHATES

E. S. CANELLAKIS, M. E. GOTTESMAN* AND H. O. KAMMEN

Department of Pharmacology, Yale University, New Haven, Conn. (U.S.A.)

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SUMMARY

A method is described which permits the synthesis of ribonucleoside-, and deoxyribonucleoside-triphosphates in good yields and in amounts commensurate with their use in tracer experiments.

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* A portion of the present work shall be presented as part of a research thesis in partial fulfillment of the requirements for the Degree of Doctor of Medicine according to the regulations of the Yale Medical School.

INTRODUCTION

We wish to present an enzymic method which permits the net synthesis of nucleoside triphosphates. This method uses enzyme systems isolated by simple fractionation procedures from *E. coli* B and from brewer's yeast. The specificity and the activity of the enzyme systems are wide enough to permit the synthesis of both ribonucleoside and deoxyribonucleoside triphosphates in relatively large amounts commensurate with the use of these compounds as substrates for the study of polymerization and other reactions.

METHODS AND RESULTS

E. coli *B Enzyme*

The growth medium contains (per l): Bactopeptone (Difco) (5 g), Bacto-yeast extract (Difco) (10 g), KH_2PO_4 (17 g), K_2HPO_4 (21.8 g), Antifoam AF (Dow Corning) (about 20 mg), and glucose (10.0 g). The glucose is autoclaved separately in 100 ml of water. The solutions, when prepared in lots of 1 l, are autoclaved for 15 min at a pressure of 15 lbs/sq. in; they are incubated for 10 min at the same pressure when prepared in 10-l lots. An initial 10 ml of bacterial culture is prepared by loop transfer from stock agar slants. After a 4- to 5-h period of incubation at 37°, the inoculum (10 ml) is poured under sterile conditions into 1 l of growth medium, which is stirred overnight at room temperature. This is then added to 10 l of growth medium, which is agitated by vigorous aeration, under sterile conditions, for 3 h at 37°. Then, when the bacteria are in the middle of the exponential phase of growth, the cells are collected by centrifugation at 1°. The cells are washed once by suspension in cold isotonic saline and centrifuged at 1° for 30 min at 30,000 × *g*. The yield is 100 ± 10 g (wet wt.) per 11 l of medium.

Disruption of the total mass of organisms is performed, in a Servall Omni-Mixer, by blending at top speed in the presence of 50 ml of 0.01 *M* potassium phosphate buffer (pH 7.4), and 250 g of glass beads* for 15 min at 0°. After the addition of another 250 ml of the cold phosphate buffer, the disruption in the Omni-Mixer is repeated for 15 min more. The mixture is then centrifuged for 30 min at 30,000 × *g* and the precipitate discarded. (In some experiments, in which the precipitate was re-extracted with 0.01 *M* potassium phosphate buffer, an appreciable amount of enzyme activity was recovered.) To 100 ml of the supernatant solution are added 4 ml of 1 *M* potassium phosphate buffer (pH 8.0), and the mixture is incubated for 1 h at 37°**. After centrifugation, ammonium sulfate is added to the supernatant solution to 55% saturation (35.2 g/100 ml) and the suspension is allowed to equilibrate for 30 min

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Abbreviations used in text: AMP, GMP, CMP, UMP represent the corresponding 5'-monophosphates of adenosine, guanosine, cytidine and uridine. dAMP, dGMP, dCMP, dTMP represent the 5'-monophosphates of deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine. ATP, adenosine triphosphate; Tris, tris(hydroxymethyl) aminomethane; PRPP, 5'-phosphoribosyl-1'-pyrophosphate; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; CTP, cytidine triphosphate; UTP, uridine triphosphate.

* Glass type superbrite No. 100, 200 microns average diameter, Minnesota Mining Company, Reflective Products Division, St. Paul, Minnesota. We are indebted to Dr. S. Ochoa of New York University for information concerning this method. The glass beads are washed with water, ethyl alcohol and acetone before use.

** Personal communications from Dr. Paul Berg, now of Stanford University.

in an ice bath. The suspension is then centrifuged at 30,000 × g for 30 min; it is necessary to centrifuge at high speeds in order to obtain a precipitate of minimum volume. The precipitate is dissolved in 20 ml of 0.1 *M* potassium phosphate buffer (pH 8.0); this results in a solution of pH 6.8–7.0. This solution is stirred in a water bath of 65° until its temperature reaches 50° and it is then transferred to a bath of 50° and kept there for 90 sec. The suspension is immediately cooled to 0° in an ice-bath, and is centrifuged at 1° for 10 min at 30,000 × g. The precipitate is discarded and the supernatant fluid is dialyzed overnight against two 4-l changes of 0.1 *M* potassium phosphate buffer (pH 7.4), or against two 4-l changes of 0.05 *M* potassium glycinate buffer (pH 8.1), when it is used for the synthesis of CTP from orotic acid.

Brewer's yeast enzyme

The method of preparation of this enzyme is identical to that used by LIEBERMAN *et al.*[1] for the preparation of orotidylic acid pyrophosphorylase through the alcohol precipitation step. The alcohol precipitate, obtained from an original extract of 30 g of brewer's yeast, is dissolved in 10 ml of 0.1 *M* potassium phosphate buffer (pH 7.4), and dialyzed overnight in the same way and with the same qualifications as described above for the *E. coli* enzyme.

Both enzyme preparations have been stored at −20° for periods up to 6 months without appreciable loss of activity. The precipitate which occurs upon freezing and thawing does not affect the total enzyme activity. Creatine kinase was prepared according to the short method of KUBY, NODA AND LARDY[2], but was not crystallized. The creatine phosphate and the nucleosides and nucleotides used in this study were commercial preparations. The PRPP used for the synthesis of UTP was prepared according to the method of KORNBERG *et al.*[3] and purified with charcoal[9]. The high content of inorganic phosphate in this preparation inhibits the synthesis of CTP[4]; for preparation of the latter from orotic acid the commercial magnesium salt of PRPP was employed.

Column chromatography

At the end of the incubation period, the incubation mixture is diluted 3- to 4-fold with water and and is transferred quantitatively to a column of Dowex-1-10X formate resin (20 cm × 1 cm); after addition of the sample the column is washed with 20 to 30 ml of water. This procedure permits the removal of the protein without acid deproteinization and avoids losses of triphosphates which might occur with the latter method. It also avoids overloading of the column with large amounts of anions which might accumulate if acid deproteinization were used.

The products of the enzymic reaction are separated by column chromatography using the ammonium formate system of pH 4.25 which was previously described[5]. This system, in addition to the fine separation of the deoxynucleoside-5′-monophosphates, affords excellent separation of the nucleoside mono-, di- and triphosphates involved in these studies. The peaks tend to be narrow and well-defined and emerge more rapidly than when ammonium formate solutions of pH 5.0[6] are employed. With the ammonium formate system of pH 4.25, GTP appears immediately after ATP. When the incubation mixtures contain large amounts of salts, elution of all nucleoside triphosphates may be accomplished best with 1 *M* ammonium formate in the reservoir. When small amounts of salts are present, it is necessary to change to 2.0 *M* ammonium formate of pH 4.25 after approximately 100 fractions of 5 to 6 ml each have been eluted. Under the latter conditions, elution of GTP is usually completed when 150 to 170 fractions have been collected.

Isolation of nucleoside triphosphates

The fractions corresponding to the individual nucleoside triphosphates are pooled, adjusted to pH 5 to 7, diluted 5- to 10-fold and passed through a column of Dowex-1-chloride (1 cm × 1 cm). The column is washed with 10 ml of water and with 6 ml of 0.1 *M* NaCl to remove excess formate and other contaminants and is then eluted with 3 *M* NaCl until the nucleoside triphosphates are removed. After dilution of the eluate to 12 ml, in an ice-bath, the pH is adjusted to 8.5 and 1.0 ml of 1.3 *M* $Ba(ClO_4)_2$ is added. (The latter is prepared by neutralization of $HClO_4$ with $Ba(OH)_2$ to pH 8.5). To this cold solution, 2.5 volumes of cold 95 % ethanol are added and the barium salts of the nucleoside triphosphates are allowed to precipitate over a period of 2 to 3 h. The barium salts are then collected, washed first with alcohol and then with ether, and dried.

The sodium salts of the nucleoside triphosphates may be obtained by dissolving the corresponding barium salts in 0.1 *N* HCl, precipitating the barium as $BaSO_4$ with sodium sulfate, and neutralizing the supernatant solution. An alternative procedure is to stir a suspension of the barium nucleoside triphosphates with about 1 ml of Dowex-50-H^+-8X or Dowex-50-Na^+-8X for 15 min in an ice-bath. (Before using this resin it should be thoroughly washed with water until the pH of the eluate is about 5.) The supernatant solution is then collected, the resin washed with water, and the combined solutions neutralized with the appropriate base. Occasionally the resin eluate may contain traces of barium, which can be removed as barium sulfate. Yields of the sodium nucleoside triphosphates during the isolation procedure, from the column fractions to the sodium salts, are generally from 85 to 90 %.

TABLE I

PRODUCTS OF ENZYMIC REACTION

Substrate	*Enzyme*	*% of Substrate*			*Acid-Labile P: Total P*
		Mono-P	*Di-P*	*Tri-P*	
dAMP*	*E. coli* B			58	2.38:3.17
dCMP	*E. coli* B	12–18	9	58–68	2.01:3.22
dTMP	*E. coli* B	77	—	22	—
dGMP	*E. coli* B	—	9	75	2.18:2.96
AMP	*E. coli* B	2	9	89	2.15:3.15
GMP	*E. coli* B	3	6	91	2.18:3.13
UMP	*E. coli* B	3	3	90–95	2.02:3.20
CMP	*E. coli* B	20–41	4–7	51–70	2.02:3.24
Guanosine	*E. coli* B	4	8	88	2.15:3.05
Orotic acid	Brewers yeast	UMP	UDP	UTP	2.03:3.17
		5	5	90	
Orotic acid	Brewers yeast plus *E. coli* B	UMP	UDP	UTP	—
		—	—	14	
		CMP	CDP	CTP	2.02:3.24
		—	—	61	

* The dATP fraction is not separated from ATP. The yields of dATP have been corrected for the ATP added. Should selective removal of ATP in a mixture of ATP and dATP be desired, this may be accomplished by the method of LEHMAN *et al.*[12], or by that of KLENOW AND LICHTLER[15].

Identification of the final products was based on the ultraviolet spectral data[7,8], position of the eluted peaks on chromatograms (as compared with those of known samples), and by determination of the content of acid-labile and total phosphate. The incubation conditions, the yields of mono-, di- and triphosphates, and the phosphate analyses of the triphosphates synthesized, are summarized in Table I.

Incubation conditions

Substrates: dCMP, dTMP, UMP, CMP, AMP. Total volume, 5.0 ml, contained: substrate 15.9 to 19.9 µmoles, $MgCl_2$ 67 µmoles, ATP 6.4 µmoles, creatine phosphate 77.1 µmoles, creatine kinase 400 µg, Tris buffer (pH 7.4) 200 µmoles, 1.0 ml of enzyme. Incubation: 20 min at 38°.

Substrates: dAMP, dGMP. Total volume, 5.0 ml, contained: Substrate 15.9 to 17.1 µmoles, $MgCl_2$ 67 µmoles, ATP 1.6 µmoles, creatine phosphate 123.3 µmoles, creatine kinase 400 µg, Tris buffer (pH 7.4) 200 µmoles and 1.0 ml of enzyme. Incubation: 40 min at 38°.

Substrates: guanosine, GMP. Incubation conditions were the same as for dAMP and dGMP, except that potassium phosphate buffer (pH 7.4) was used in place of Tris buffer.

Substrate: orotic acid

(1) CTP Synthesis. Total volume, 7.6 ml, contained: orotic acid 10 µmoles, MgPRPP 14 µmoles, $MgCl_2$ 50 µmoles, NH_4Cl 50 µmoles, ATP 6.4 µmoles, creatine phosphate 77.1 µmoles, creatine kinase 500 µg, potassium glycinate (pH 8.1) 800 µmoles and 2.0 ml each of brewer's yeast enzyme and *E. coli* enzyme (both dialyzed against 0.05 *M* potassium glycinate (pH 8.1). Incubation: 60 min at 38° (after 30 min of incubation an additional 0.4 ml of solution was added: this contained ATP 3.1 µmoles, creatine phosphate 30.8 µmoles and creatine kinase 200 µg).

(2) UTP Synthesis. For the synthesis of UTP, the NH_4Cl and *E. coli* enzyme were omitted and PRPP, prepared as described[3,9], was used in place of the commercial MgPRPP.

DISCUSSION

The objective of this study was to devise a simple method, which would supplement the methods available in the literature[5,9-13] for the synthesis of radioactive ribonucleoside and deoxyribonucleoside triphosphates from starting materials which are more easily obtainable in radioactive form. The $[^{14}C]$ribonucleoside- and $[^{14}C]$-deoxyribonucleoside-5'-monophosphates are readily prepared by the action of venom diesterase[14] on the respective $[^{14}C]$nucleic acids of *E. coli* grown on $^{14}CO_2$. RNA and DNA, uniformly labeled with ^{14}C, are also commercially available. $[^{14}C]$guanosine may be obtained either commercially, or by the action of intestinal phosphatase on $[^{14}C]$guanylic acid (2',3'); the latter may be prepared by alkaline hydrolysis of $[^{14}C]$-RNA. The broad specificity of the enzyme preparation from *E. coli* permits the conversion of guanosine and the nucleoside-5'-monophosphates to the corresponding triphosphates in high yield; however, dTMP is more slowly phosphorylated. Nevertheless, the low phosphatase content of the preparation permits the use of prolonged incubation periods when larger amounts of product are desired.

Uridine, cytidine and adenosine cannot serve adequately as precursors of the corresponding triphosphates with the *E. coli* enzyme, since the enzyme contains powerful uridine nucleosidase and cytidine and adenosine deaminase activities. Thymidine is extensively phosphorolyzed by this enzyme. An alternative approach to the synthesis of labeled pyrimidine ribonucleoside-5'-triphosphates involves the use of [^{14}C]orotic acid as substrate with the brewer's yeast enzyme, either alone for the preparation of UTP, or in combination with the *E. coli* enzyme for the preparation of CTP. The *E. coli* enzyme contains the UTP aminase described by LIEBERMAN[4] and, when it is coupled with the brewer's yeast system in the presence of NH_4^+, orotic acid is extensively converted to CTP. Since phosphate and tris are inhibitory to the amination of UTP[4], the preparation of CTP by this method is performed with enzymes dialyzed against 0.05 *M* potassium glycinate (pH 8.1).

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THE EFFECT OF DARK, AEROBIC GROWTH ON THE PHOTOSENSITIVITY OF CAROTENOIDLESS *RHODOPSEUDOMONAS SPHEROIDES*

MARTIN DWORKIN

Department of Microbiology, Indiana University Medical Center,
Department of Basic Sciences, Indiana University School of Dentistry, Indianapolis, Ind. (*U.S.A.*)

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SUMMARY

A study has been made of the protective effect of prior, dark aerobic growth on the photosensitivity of a carotenoidless mutant (strain U.V. 33) of *Rhodopseudomonas spheroides*. The protection could not be related to any of the following factors: (a) Dilution of chlorophyll by aerobic growth. (b) Migration of chromatophores. (c) Change in the nature of the chlorophyll spectrum. (d) Aerobic synthesis of catalase. (e) Metabolic utilization of oxygen.

INTRODUCTION

Griffiths and Stanier[1] described a carotenoidless mutant of the photosynthetic bacterium, *Rhodopseudomonas spheroides*. When this mutant (UV 33) is exposed simultaneously to visible light and air there are two gross manifestations. The intracellular chlorophyll is destroyed and the cells are killed. The photosensitizing pigment has been identified as the intracellular chlorophyll[2].

During a more detailed investigation of this phenomenon, the prior growth conditions of the inoculum were shown to affect the course of the photosensitization[3]. *R. spheroides* can be grown photosynthetically (under anaerobic conditions) or heterotrophically (under aerobic conditions). If UV 33 is grown aerobically in the dark for a period of time which may be as short as one half a generation time the photosensitized killing is preceded by a lag period (Fig. 1). The length of the lag period seems to be a function of the extent of the prior, aerobic growth. The maximal killing rate finally obtained is slightly lower than that obtained with the control cells (Fig. 1).

The purpose of this investigation was to attempt to interpret the desensitization in terms of the physiological changes involved in the transition from photosynthetic to heterotrophic growth.

MATERIALS AND METHODS

Methods for the maintenance, cultivation and irradiation of UV 33 have been presented elsewhere[3].

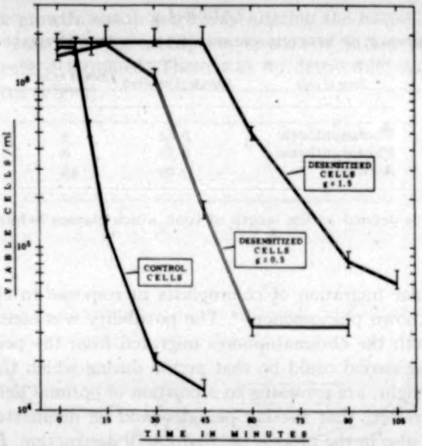

Fig. 1. The effect of dark, aerobic growth on photokilling. Control cells were grown photosynthetically. Desensitized cells (g = 0.5 and g = 1.5) were grown photosynthetically and then were grown aerobically in the dark for 0.5 and 1.5 generations respectively.

Chlorophyll determinations were performed according to the method of COHEN-BAZIRE *et al.*[4]. The chlorophyll content of the cell is expressed as the specific chlorophyll $\left(\frac{\text{mg chlorophyll/100 ml culture}}{\text{O.D.}_{680\,\text{m}\mu}\text{ of the culture}}\right)$.

The spectra of intact cells were determined by the method of LATIMER[5]. Cells were suspended in a 42 % solution of bovine serum albumin and examined with a Beckman Model DU spectrophotometer.

Catalase was assayed by exposing cells to hydrogen peroxide at 4° over a measured interval of time (t, in minutes). The initial peroxide concentration (x_0) and the final concentration (x) were determined by oxidation with potassium permanganate. The rate constant was divided by the O.D. (at 680 mμ) of the culture to give an expression of the specific catalase of the cells $K = \frac{\log_e x_0 - \log_e x}{t\,\text{O.D.}_{680\,\text{m}\mu}}$. This is a modification of the method of BONNICHSEN *et al.*[6].

Light intensity was measured with a Photovolt Photometer. The cells were photosensitized at an intensity of 1000 foot candles.

RESULTS AND DISCUSSION

These organisms do not synthesize pigment during aerobic growth. It was necessary, therefore, to rule out the possibility that aerobic growth resulted in a dilution of pigment, sufficient to prevent photosensitization. COHEN-BAZIRE *et al.*[4] have shown that the chlorophyll concentration of *R. spheroides* is an inverse function of the light intensity during photosynthetic growth. It was therefore possible, by manipulating the light intensity, to grow cells photosynthetically with a chlorophyll content as low as that of desensitized cells. These cells showed a normal sensitivity (Table I).

TABLE I

EFFECT OF SPECIFIC CHLOROPHYLL ON DESENSITIZATION

State of cells	*Specific chlorophyll*	*Length of lag (min)**
Photosynthetic	0.84	5
Photosynthetic	0.30	8
Aerobic	0.29	45

* The lag period is defined as the length of time which elapses before the maximal killing rate is achieved.

The intracellular migration of chloroplasts in response to changes in light intensity is a well known phenomenon[7,8]. The possibility was considered that during dark, aerobic growth the chromatophores migrated from the periphery of the cell. In this case the lag period could be that period during which the chromatophores, in response to the light, are returning to a position of optimal light receptivity. One would expect, therefore, that the lag period would be manifested not only in the killing process but also in the process of chlorophyll destruction. Photosynthetic and desensitized cells were irradiated aerobically and the rate of chlorophyll destruction was measured. There was no lag period in either photosynthetic or desensitized cells (Fig. 2). The chromatophores are, therefore, in a position of light receptivity during the lag period.

SHIBATA has shown that incubation of intact leaves in the dark can cause a reversible conversion between chlorophyll and protochlorophyll[9]. To test the possi-

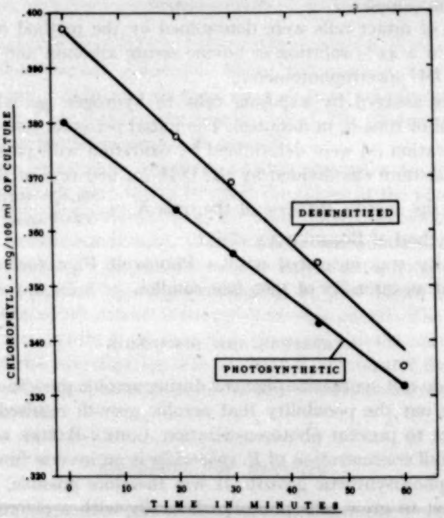

Fig. 2. Chlorophyll destruction in control (photosynthetic) and desensitized cells.

bility that the dark growth was in some way altering the nature of the chlorophyll or the chlorophyll complex, the absorption spectra of intact photosynthetic and desensitized cells were determined. There was no detectable, significant difference between their spectra (Fig. 3).

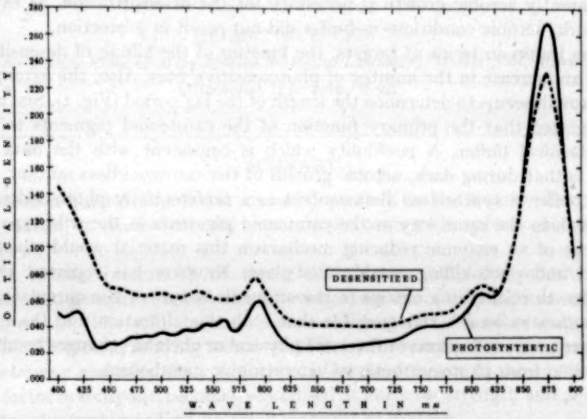

Fig. 3. Absorption spectra of intact photosynthetic and desensitized cells.

CALVIN has suggested that the formation of reactive peroxides may play a role in the photosensitization process[10]. The adaptive synthesis of catalase as a response to aerobic conditions could, therefore, have a protective function. Photosynthetically grown cells were found to be devoid of measurable catalase. After a brief exposure to dark, aerobic conditions catalase was rapidly synthesized by the cells. Desensitization, however, did not take place until the cells had started growing aerobically (Table II). In view of the separation in time between adaptive catalase synthesis and desensitization it is unlikely that the two are causally related.

The photosensitization is an oxidative process. If dark, aerobic growth results in an adaptation to the metabolic use of oxygen as a terminal hydrogen acceptor it is conceivable that this would preclude the use of oxygen in a photooxidative process. The lag would, in this case, be the result of endogenous metabolic activity

TABLE II

RELATION BETWEEN SPECIFIC CATALASE AND DESENSITIZATION

State of cells	*Specific catalase*	*Length of lag (min)*
Photosynthetic	0	5
Photosynthetic and exposed to dark aerobic growth conditions for 90 min*	0.73	8
Aerobic	0.80	50

* An adaptation period was necessary for the transition from photosynthetic to heterotrophic growth. During this 90 min period, no growth took place.

and should be affected by low temperatures. It has already been shown that the photokilling itself is not affected by temperatures between 4° and 40°[3]. Irradiating desensitized cells at 1° did not shorten the length of the lag period. It is unlikely, therefore, that the protection is a direct result of the aerobic metabolism of the cell.

Apparently aerobic growth is necessary for the desensitization, as exposure of cells to dark, aerobic conditions in buffer did not result in protection.

If one thinks in terms of targets, the kinetics of the killing of desensitized cells indicates an increase in the number of photosensitive sites. Also, the extent of dark, aerobic growth seems to determine the length of the lag period (Fig. 1). SISTROM *et al.*[2] have suggested that the primary function of the carotenoid pigments is to act as a photochemical buffer. A possibility which is consistent with the data thus far obtained is that during dark, aerobic growth of the carotenoidless mutant, a photochemical buffer is synthesized that can act as a preferentially photooxidizable substrate, much in the same way as the carotenoid pigments in the wild type cells. In the absence of an enzymic reducing mechanism this material would eventually be exhausted and photokilling would take place. NEWTON has reported that dark, aerobic growth results in a change in the antigenic nature of the chromatophores of *Rhodospirillum rubrum*[11]. It is possible that both this alteration and the temporary desensitization are reflections of the same physical or chemical changes resulting from the transition from photosynthetic to heterotrophic metabolism.

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THE ACTION OF RELAXING FACTOR ON ACTOMYOSIN*

HELMUT MUELLER**

Institute for Muscle Research at the Marine Biological Laboratory, Woods Hole, Mass. (U.S.A.)

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SUMMARY

1. The actions of the relaxing factor are not dependent on the structural organisation of the myofibril; it reacts with the most simple contractile system: actomyosin + ATP + ions.

2. The specific interaction of the relaxing factor with the actomyosin-ATP system has been demonstrated by the following reactions: (a) Synaeresis and ATPase are inhibited. (b) The ATP induced synaeresis (contraction) of actomyosin-gel is reversed by the system relaxing factor + ATP. (c) All reactions show a characteristic ATP dependence and sensitivity to Ca^{++}. (d) In the presence of the system ATP + relaxing factor actomyosin becomes solubilised at low ionic strength and precipitated in the form of a synaeresed gel by small amounts of calcium.

INTRODUCTION

In 1952 MARSH[1] reported the presence of a factor in muscle extract which inhibits the ATP induced contraction of myofibrils. Later it was shown[2], that the RF activity resides in the microsomal fraction of muscle extract. Similar conclusions had been reached earlier by EBASHI[3-5]. The action of this factor has been studied by BENDALL[7,8] and WEBER AND HASSELBACH[9]. They described the following properties: (a) Inhibition and reversal of ATP induced synaeresis of myofibrils. (b) Inhibition of ATPase activity. (c) Inhibition of isometric tension and isotonic shortening. (d) Requirement of higher ATP concentrations than those necessary for the contractile response. (e) Ca^{++} abolishes all actions (a–d).

The present communication is concerned with the question of whether these characteristic phenomena can also be demonstrated with the most simple and chemically defined contractile system: purified actomyosin, or whether the relaxation process is dependent on the organised structure of the striated myofibril. (All of the above criteria were examined except (c).)

This "MARSH Factor" has been differently named by various authors. In the present communication the term Relaxing Factor (RF) will be used, without implication as to its chemical individuality or possible complexity.

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[*] This work was done during the tenure of an Advanced Research Fellowship of the American Heart Association.

[**] Present Address: Department of Biochemistry, University of Birmingham, England.

MATERIALS AND METHODS

Relaxing factor

Two types of preparations were used: (a) Dialysed muscle extract. (b) The resuspended microsomal fraction of muscle extract.

In both preparations the procedure described by PORTZEHL[2] was essentially followed. The muscles of freshly killed rabbits were cooled and then homogenised with 3 volumes of extraction solution: 0.05 *M* KCl, 0.02 *M* imidazole buffer, 0.005 *M* K-oxalate, pH 7.2. Homogenisation was performed by Waring blendoring at 0° for 1.5 min; the homogenate was then stored in ice for 15 min and again blendored for 1.5 min. The residue and the larger particles of the extract were removed by 25 min centrifugation with 12,000 × *g* at 0°.

Preparation (a) was obtained by dialysing the extract against a solution of 0.05 *M* KCl, 0.005 *M* $MgCl_2$, 0.005 *M* K-oxalate, 0.02 *M* imidazole, buffer, pH 7.2. For preparation (b) the extract was centrifuged for 2 h at 45,000 × *g*, the supernatant poured off, and the microsomal sediment resuspended by means of a glass homogeniser in a volume of 0.1 of the original extract. For this suspension the same solution used for dialysis was employed. Larger and aggregated particles were removed by centrifuging this microsomal resuspension at 12,000 × *g* for 20 min at 0°.

Prior to each experiment the pH of both preparations was adjusted to 6.5.

Actomyosin

SZENT-GYÖRGYI'S[10] method was followed for the preparation of "Myosin B". Fresh, cooled and ground muscle was extracted with 3 volumes of 0.6 *M* KCl at pH 9 (Weber Edsall solution) for 24 h at 0°. The insoluble residue was removed by centrifugation, and the actomyosin precipitated by diluting the extract with 12 volumes of cold distilled water. The precipitate was dissolved in 0.6 *M* KCl. Purification was achieved by 3 further reprecipitations. Prior to the experiments, the final actomyosin solution was again precipitated by diluting with 12 volumes of cold water and brought to a concentration of about 6 mg/ml by centrifugation. A uniformly appearing gel was thus obtained, which was stored at 0° not longer than 3 days.

Solvents

All experiments were carried out in solubions of the following ionic composition: 0.05 *M* KCl, 0.005 *M* $MgCl_2$, 0.005 *M* K-oxalate, 0.02 *M* imidazole buffer, pH 6.5. The effect of calcium was tested by adding 2 μmoles/ml of $CaCl_2$ to this solution.

ATP

ATP was obtained as disodium salt from Pabst Chemical Company, Milwaukee. It was dissolved in the described solvent and the pH adjusted to 6.5.

Synaeresis experiment

Concentration series of our standard relaxing factor preparations were made by progressive dilution with the solvent. Then, 2.5 ml of the desired concentrations were pipetted into graduated centrifuge tubes (Kolmer type) and 2.5 ml of actomyosin-gel were added to each tube and mixed by inversing the tubes several times. All steps were performed in an icewater bath. The tubes were then placed into centrifuge cups, which were filled with water at 18°, balanced and permitted to equilibrate. Then

0.5 ml of ATP solution was added and the tubes were centrifuged for 3 min at 2,800 rev./min, at room temperature. Synaeresis occurred during this time and was measured by the volume of the sedimented actomyosin-gel.

RESULTS

Effect of RF concentration on ATP-induced synaeresis of actomyosin

The photographic record of a representative experiment is shown in Fig. 1A. All tubes contain the same amount of actomyosin (15 mg) and the same concentration of ATP (3 µmoles/ml), but varying concentrations of RF. In tube 1 the standard microsomal resuspension is diluted 8 times, then 16, 32, 64,... 1024 times. Tube 9 serves as a control; it contains no RF and shows maximal synaeresis of the actomyosin-gel. In tubes 7 and 8 with RF dilutions of 512 and 1024, ATP also causes maximal synaeresis. Inhibition of synaeresis begins with an RF dilution of 256 (tube 6) and increases with rising RF concentration. With the highest RF concentration (tube 1) no synaeresis occurs; rather a "solubilisation" of the actomyosin gel is noted. It does not sediment in the centrifugal field employed, and appears as a homogeneous, moderately viscous, liquid. Part of the actomyosin is also solubilised in the tubes with lower RF concentrations.

As the ATP concentration decreases, the solubilised actomyosin precipitates again, when the tubes are kept at room temperature for some time. It can be shown that the amount of actomyosin which was solubilised varies with the RF concentration.

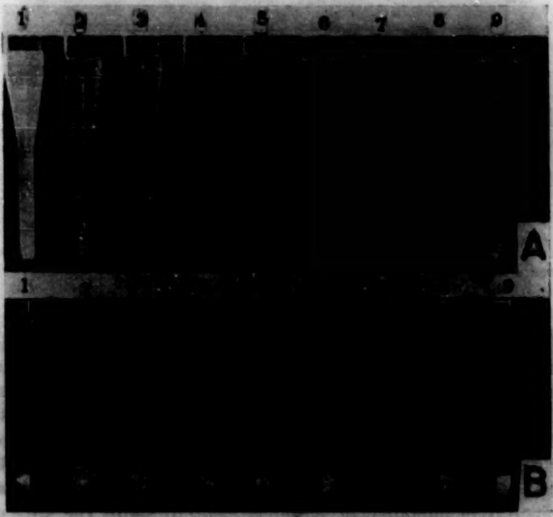

Fig. 1. Effect of varying concentrations of RF on ATP induced synaeresis of actomyosin. (A) without Ca^{++}; (B) in presence of Ca^{++}. Tubes 1–8 contain RF dilutions of from 8 to 1024 times. Tube 9 contains no RF.

Fig. 1B shows the same experiment in the presence of $CaCl_2$ (less than 1 µmole/ml of free Ca^{++}). All factor activity appears abolished, and the same maximal contraction of the actomyosin-gel is noted in all tubes, regardless of the concentration of RF present.

A graphical representation of this type of experiment is shown in Fig. 2. Curve 1 was obtained by progressive dilution of muscle extract. At a dilution of 64 times, it still significantly inhibits synaeresis and has, therefore, a RF concentration of 64 units/ml. One unit of RF activity is defined as the smallest quantity of RF which, when present in 1 ml of the reaction volume, still causes significant inhibition of synaeresis (as evidenced by an actomyosin volume of at least 3 times the volume of the control).

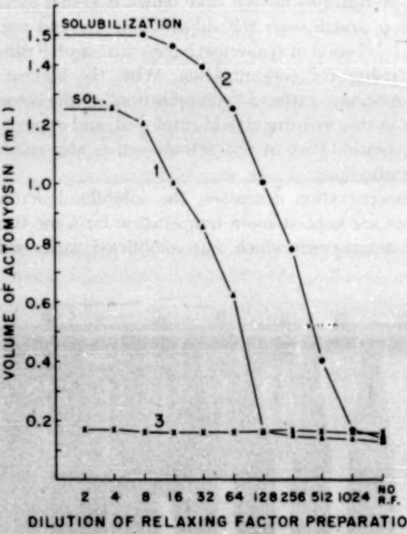

Fig. 2. Effect of varying concentrations of RF on ATP induced synaeresis of actomyosin. (1) Dilution of dialysed muscle extract; (2) dilution of resuspended microsomal fraction; (3) experiment in presence of Ca^{++}.

Effect of ATP concentration on RF activity

A representative experiment is photographed in Fig. 3. Tube 1 contains no ATP; in tubes 2–6 ATP is present in 0.2, 0.5, 1.0, 2.0 and 3.0 m*M* concentration. Fig. 3A shows the control experiment, without RF. Practically maximal synaeresis occurs with all ATP concentrations employed. Fig. 3B shows the same experiment with an RF concentration of 30 units/ml present in all tubes. With 0.2 m*M* ATP, the RF shows no effect. With rising ATP concentrations, the same concentration of RF causes increasing inhibition of synaeresis. Fig. 3C shows the same experiment in the presence of RF + Ca^{++}; again all RF activity is abolished, regardless of the ATP concentration employed.

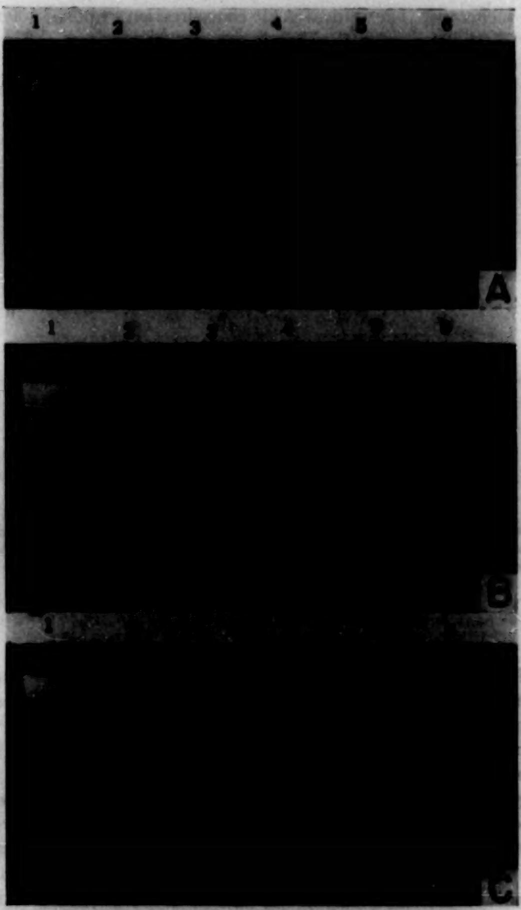

Fig. 3. Effect of varying ATP concentrations on RF activity. (A) Control experiment in absence of RF; (B) relaxing factor present in all tubes; (C) RF + Ca^{++} present in all tubes. Tube 1 contains no ATP. Tubes 2–6 contain 0.2, 0.5, 1.0, 2.0, and 3.0 m*M* ATP.

Fig. 4 presents a series of experiments, (such as illustrated in Fig. 3) each with constant RF concentration, showing the effect of varying ATP concentrations. The numbers at the right of the curves express the RF concentration in the respective experiments. All ATP concentrations in the control experiment ("No RF") cause maximal synaereisis. It is clearly evident that with low ATP concentrations, still sufficient to cause maximal synaeresis in the control experiment, the relaxing factor is relatively ineffective. The critical ATP concentration required for the action of the relaxing factor decreases with rising factor concentration.

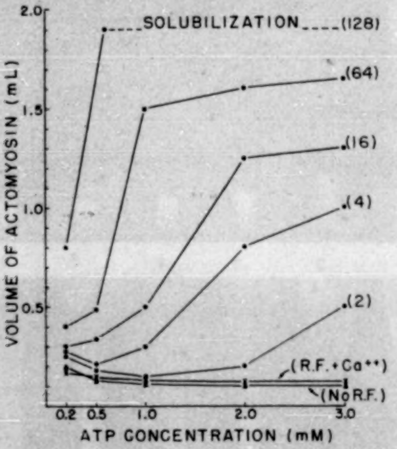

Fig. 4. Relationship between ATP and RF concentrations. Numbers on the right side of curves express the RF concentration. The lower curves are control experiments in the absence of RF and in the presence of RF (128 units/ml) + Ca^{++}, (For further explanation see text.)

Rehydration (expansion) of synaeresed actomyosin-gel by RF + ATP

While the described experiments have demonstrated that the ATP-induced synaeresis of actomyosin can be inhibited by the relaxing factor, it remains to be shown that the system RF + ATP is able actively to reverse the synaeresis of an actomyosin-gel, previously contracted by ATP.

The actomyosin-gel used in these experiments was synaeresed with 0.6 µmole/ml of ATP, then washed with standard solvent to remove the nucleotides. This washing caused no significant change in the hydration of the synaeresed actomyosin. With this presynaeresed actomyosin, experiments as illustrated in Fig. 5 were performed. (A) The control experiment in the absence of RF. All tubes contain the same quantity of actomyosin. Tube I contains no ATP. In tubes 2–6 ATP is present in 0.2, 0.5, 1.0, 2.0 and 3.0 m*M* concentration. Since the actomyosin is presynaeresed, rising ATP concentrations cause only a minimal amount of further synaeresis. (B) The same experiment in the presence of relaxing factor. Beginning with 1 m*M* ATP concentration, an increasing rehydration of the synaeresed actomyosin-gel is noted. With 3.0 m*M* ATP it is about twice the original volume. (C) In presence of Ca^{++} + RF no rehydration occurs. Essentially the same experimental pattern as that obtained without relaxing factor is apparent.

Fig. 6 shows a similar experiment in graphical representation. Curve 1 represents the control experiment without RF. Curve 2 shows the re-expansion of presynaeresed actomyosin in the presence of RF + ATP. The inability of this system to exert any effect in the presence of Ca^{++} is apparent from Curve 3.

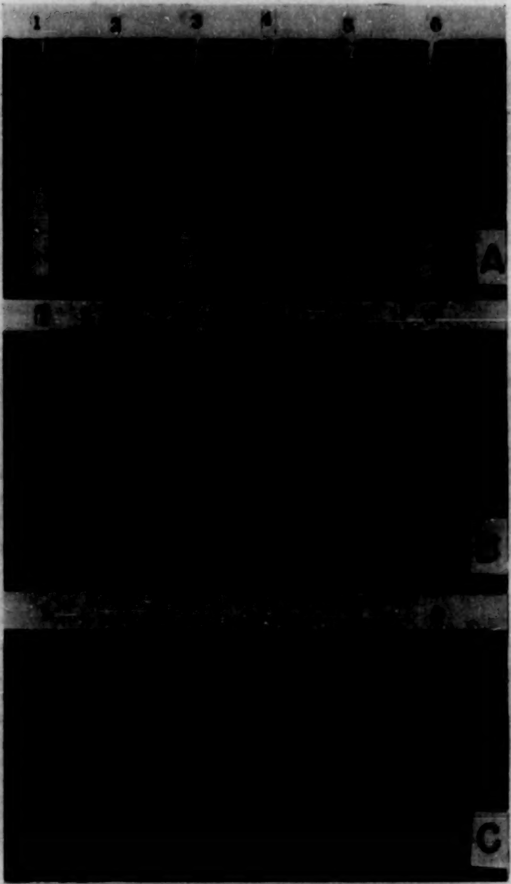

Fig. 5. Expansion of pre-synaeresed actomyosin gel. (A) Control experiment in the absence of RF. (B) Relaxing factor present in all tubes. (C) Control experiment in presence of Ca^{++} + RF. Tube 1 contains No ATP. Tubes 2–6 contain 0.2, 0.5, 1.0, 2.0, and 3.0 mM ATP.

The RF concentration in these experiments is only about 20 units/ml. With higher RF concentration, swelling of the synaeresed actomyosin-gel can be further increased.

Effect of RF on the ATPase of actomyosin-gel

Experimental Procedure: 1 ml of actomyosin-gel and 1 ml of the relaxing factor preparation were mixed and equilibrated at 23°. Then 1 ml of ATP solution was

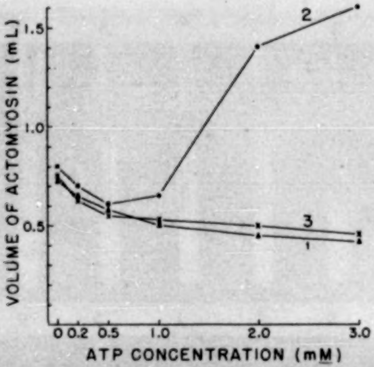

Fig. 6. Expansion of presynaeresed actomyosin-gel. (1) Control experiment in absence of RF. (2) Rehydration in presence of RF. (3) Control experiment in presence of RF + Ca^{++}.

added, and hydrolysis stopped after 3 min by addition of 1 ml of 10 % trichloracetic acid. Each determination was accompanied by a control with trichloracetic acid addition at zero time. Liberation of phosphate was expressed as the difference between these determinations. The FISKE AND SUBBAROW method was used for phosphate determination. In parallel experiments the ATPase of the same quantities of actomyosin and relaxing factor were measured separately, under identical conditions.

The volume of the reaction mixture was 3 ml, containing 9 μmoles of ATP and 5 mg of actomyosin. The RF concentration in the reaction mixture was chosen, on the basis of synaeresis experiments, to be about 15 times the minimal active concentration. The ionic composition of the reaction mixture was identical with that of the synaeresis experiments: 0.05 M KCl, 0.02 M imidazole buffer, 0.005 M $MgCl_2$, 0.005 M K-oxalate.

Table I shows the results of five experiments reflecting a representative cross section of our experience with different RF and different actomyosin preparations.

TABLE I

INHIBITION OF ATPASE OF ACTOMYOSIN GEL BY RELAXING FACTOR

The experimental data show liberation of phosphate in each experiment. AM: Liberation of phosphate by actomyosin. RF: Liberation of phosphate by the relaxing factor. AM + RF: Liberation of phosphate by the combined system. Inhibition indicates Ratio:

$$\frac{\text{Phosphate liberation by AM in presence of RF}}{\text{Phosphate liberation by AM in absence of RF}}$$

Experiment No.	*1*	*2*	*3*	*4*	*5*
	μmoles of phosphate liberated (3 min)				
AM	2.34	2.11	3.95	1.90	3.13
RF	0.25	0.80	0.40	1.31	1.28
AM + RF	0.72	1.29	0.91	1.58	2.00
AM in presence of RF (AM + RF) — (RF)	0.47	0.49	0.51	0.27	0.72
Inhibition	0.20	0.23	0.13	0.14	0.23

The relaxing factor contains the granula-bound ATPase of KIELLEY AND MEYERHOF[11]. The activity of this enzyme shows considerable variability in different preparations, and even in the same preparation when determinations are made some time apart. No correlation is apparent between the relaxing factor activity and the ATPase activity of the preparations. The relaxing factor in experiments 1–3 was dialysed muscle extract and in experiments 4–5, washed, resuspended microsomes.

In all experiments the presence of relaxing factor caused a strong ATPase inhibition. This was calculated to be about 80 % of the actomyosin ATPase activity, when determined separately.

In the presence of less than 1 m*M* Ca^{++}, no such inhibition occurs. On the contrary, varying degrees of activation of the actomyosin-ATPase by the relaxing factor are noted in most experiments. Table II shows three representative experiments. The relaxing factor in experiments 1 and 2 is dialysed muscle extract and in experiment 3 is washed, resuspended microsomes. Relaxing factor causes no inhibition of actomyosin-ATPase in any of the experiments; marked activation in experiments 1 and 2 and slight activation in experiment 3. The cause for this variation is not clear and deserves further study.

TABLE II

EFFECT OF Ca^{++} ON THE ATPASE INHIBITION OF ACTOMYOSIN BY THE RELAXING FACTOR

The experimental data show the liberation of phosphate in each experiment. AM ($+Ca^{++}$): Liberation of phosphate by actomyosin in presence of Ca^{++}. RF ($+Ca^{++}$): Liberation of phosphate by the relaxing factor in presence of Ca^{++}. AM + RF ($+Ca^{++}$): Liberation of phosphate by the combined system in presence of Ca^{++}. Activation indicates Ratio:

$$\frac{\text{Phosphate liberation by AM in presence of RF} + (Ca^{++})}{\text{Phosphate liberation by AM} + (Ca^{++}) \text{ in absence of RF}}$$

Experiment No.	*1*	*2*	*3*
	μmoles of phosphate liberated (3 min)		
AM ($+Ca^{++}$)	2.22	1.85	3.00
RF ($+Ca^{++}$)	1.27	0.25	2.20
AM + RF ($+Ca^{++}$)	7.00	3.48	5.80
AM in presence of RF ($+Ca^{++}$) (AM + RF) — (RF)	5.73	3.23	3.60
Activation	2.58	1.74	1.20

DISCUSSION

The present study is not concerned with the chemical composition of the RF and, in fact, no relevant conclusion can be drawn from the experiments presented. Removal of the RF-activity from muscle extract and its recovery in the microsomal sediment, as observed in our preparations, proves only that microsomes are an essential component of the relaxing factor. A considerable amount of supernatant material is attached to the microsomes.

The object of the present study has been to investigate the reactivity of the relaxing factor with the purified contractile protein actomyosin. It has been possible to reproduce in this system the essential relaxation phenomena so far observed only with striated muscle fibrils. The technique employed, however, did not permit the study of changes in tension; this remains to be demonstrated with a different technique.

Earlier, LORAND[12] had tried to show relaxation phenomena in a contractile protein system, with creatine-phospho-kinase plus creatine phosphate as relaxing factor. The modification of superprecipitation, which he observed, at an ATP concentration of 0.25 μmole/ml, was not influenced by Ca^{++}. It seems, therefore, to have no relation to the relaxation phenomena as described here. In our experimental system, creatine-phospho-kinase with creatine phosphate in the absence of microsomes was inactive.

The rehydration of synaeresed actomyosin-gel by the system RF + ATP demonstrates the conditions for the reversibility of SZENT-GYÖRGYI's[10] classical "superprecipitation". One is tempted to recall in this connection the observation of MARSH[1], that striated myofibrils, contracted by ATP, can elongate in the presence of relaxing factor + ATP. A similar "active" elongation has been demonstrated by HANSON[13] for the fibrils of the indirect flight muscle of the blow-fly, *Calliphora*. It seems reasonable to believe that swelling of a synaeresed actomyosin-gel structure, as demonstrated in the above experiments, might be related to the force behind such an elongation.

A surprising feature of these experiments was the increased solubility of actomyosin in the presence of relaxing factor and ATP, even at such a low ionic strength as that employed in our experiments. This solubility was already apparent in the preparation of the relaxing factor. On homogenisation of fresh muscle with the Waring blendor, a significant amount of actomyosin (depending on the degree of homogenisation) went into solution. On centrifugation at 12,000 × *g*, as used for the removal of the residue and the mitochondria from the extract, it did not sediment. It did, however, precipitate when the extract was kept at room temperature or dialysed at 0°, and could be resolubilised by addition of 3 μmoles/ml ATP. As shown in Figs. 1, 2 and 3, this actomyosin, solubilised at low ionic strength by relaxing factor and ATP, precipitates on addition of Ca^{++} as a synaeresed gel.

HEILBRUNN[14] has advocated for many years a theory of cellular activity and especially muscular contraction, the essential feature of which is a "protoplasmic" sol → gel transformation, caused by an intracellular Ca^{++} liberation. It seems that we have here a relatively defined protein system which is capable of performing just such a reaction. This may help to reconcile HEILBRUNN's general physiology of protoplasm with the protein chemistry of muscle.

ACKNOWLEDGEMENTS

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CONTRIBUTION À L'ÉTUDE DES MYOGÈNES DE CARPE ET DE PLIE

J. G. HENROTTE
Laboratoire de Biologie générale, Faculté des Sciences, Université de Liège (Belgique)

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SUMMARY

Contribution to the study of myogens in the carp and the plaice

On comparing the myogens of the carp, the plaice and the rabbit by electrophoresis, ultracentrifugation and fractionation respectively, a marked similarity is found between the homologous extracts of the fishes, as well as considerable difference between these and rabbit myogen. Fish myogens possess greater electrophoretic mobility, greater thermolability and higher solubility in the presence of ammonium sulphate, as well as a lower average sedimentation rate than rabbit-muscle albumins.

The group of proteins forming the main electrophoretic gradient and the group of slowly sedimenting proteins seem to be particularly characteristic of fish myogens when compared to those of the rabbit.

One albumin of the carp and two albumins of the plaice—designated as "myogens I"—were isolated and crystallized from the first group. They are distinguished by their high electrophoretic mobility and their abundance in the myogen. From the second group, another fraction called "myogen II" was isolated from carp albumin. It is characterized by very low molecular weight and a specific cyclic amino acid composition. The absence of similar proteins in the myogen of the rabbit proves that considerable differences exist between the properties of muscle albumins derived from various animal species, even though they possess similar enzymic activity.

INTRODUCTION

Les albumines du muscle strié, auxquelles on a donné le nom de myogène, représentent environ 15 % des protéines totales de ce tissu, soit 3 % du poids du muscle frais[1] et se trouvent à l'état dissous dans le sarcoplasme de la fibre[2]. On admet que la majorité d'entre elles sont des enzymes de la glycogénolyse[3]. Les myogènes de mammifères (rat et surtout lapin) sur lesquels ont principalement porté les recherches enzymatiques, seraient constitués d'une cinquantaine d'enzymes au moins[3,4]. Les myogènes des animaux d'autres classes, qui possèdent vraisemblablement une fonction et des propriétés enzymatiques analogues, n'ont par contre guère été étudiés que par la méthode d'électrophorèse.

Celle-ci montre l'existence d'une grande variabilité des propriétés électrocinétiques en fonction de l'espèce animale[5-8]. Une différence particulièrement nette est observée à ce sujet entre les myogènes de mammifères[9,10] et ceux de poissons [1,11-14] dont la vitesse électrocinétique moyenne au pH 7 est beaucoup plus élevée que celle des premiers.

Les myogènes A et B de BARANOWSKI[15] isolés du lapin sont les deux seuls constituants quantitativement importants du myogène dont le poids moléculaire soit connu: il est de 150,000[16,17]. Toutefois, les anciennes études d'ultracentrifugation de DEUTICKE[18] sur les extraits musculaires de lapin et de grenouille à faible force ionique, de même que celles plus récentes de HAMOIR[1,13] sur le myogène de carpe, indiquent l'existence d'albumines de poids moléculaires beaucoup plus faibles chez les deux dernières espèces citées.

Enfin des fractionnements systématiques des albumines musculaires n'ont guère été réalisés que chez le lapin, par précipitation au sulfate ammonique[15,19]. Citons également des études préliminaires par fractionnement à l'ethanol du myogène de perche[20].

Les grandes différences de mobilité électrophorétique des myogènes de lapin et de poisson, et l'absence d'une étude comparative approfondie de ces myogènes, nous ont déterminé à étudier par électrophorèse et ultracentrifugation les myogènes de carpe et de plie ainsi que leurs fractions et les composants cristallisés que nous en avons isolés.

MÉTHODES

Préparation du myogène

Sauf indications contraires, nos expériences ont porté sur les muscles blancs de carpes miroirs d'élevage, variété de la carpe commune (*Cyprinus carpio L.*), pesant de 1 à 2 kg. Les poissons sont tués par décapitation et les muscles immédiatement prélevés et broyés dans une machine à viande ou un "turmix". Les plies (*Pleuronectes platessa L.*) fournies par l'Institut d'Etudes Maritimes d'Ostende ont été conservées dans la glace fondante pendant environ 20 h avant prélèvement des muscles. Ceux-ci sont conservés à —20° dans des vases fermés pour empêcher la dessication, pendant un délai inférieur à 3 mois. Ils sont découpés en tranches de 40 μ d'épaisseur par un microtome automatique à congélation[21]. Les muscles des lapins, tués par ouverture de la carotide, subissent le même traitement.

La pulpe musculaire est extraite par un volume d'eau, à 4°, pendant 1 h. Après élimination des débris tissulaires par filtrage ou centrifugation, l'extrait, placé dans des tubes en cellulose, est dialysé à 2° pendant 56 h contre 2 l d'eau distillée que l'on remplace 4 fois; la résistivité finale atteint au minimum 150 à 200,000 ohms. cm.

Après élimination par centrifugation des globulines ainsi précipitées, l'extrait est généralement dialysé contre un tampon phosphatique de μ 0.15 et pH 7.3 (Na_2HPO_4, 0.029 M; NaH_2PO_4, 0.013 M; NaCl, 0.05 M) ou de μ 0.10 et pH 7.6 (Na_2HPO_4, 0.032 M et NaH_2PO_4, 0.004 M).

La concentration en protéines est mesurée au moyen d'un réfractomètre plongeant, à la température de 25° à 580 mμ (raie D du sodium).

Fractionnement du myogène

Les fractionnements au sulfate ammonique sont réalisés, au pH neutre, avec une solution saturée de sulfate ammonique de pH 7 ou à des pH acides variables, avec la même solution et du sulfate ammonique solide (réactif pour analyse) en proportion voulue pour le pH désiré. Les précipités sont séparés par centrifugation. Les teneurs en sulfate ammonique des solutions de protéines soumises au fractionnement sont exprimées en % de la teneur d'une solution saturée en sulfate ammonique à 0°, c'est-à-dire contenant 514 g de ce sel/l. La quantité de solution saturée nécessaire à ajouter à une solution donnée pour obtenir le degré de saturation voulu a été déterminée au moyen du nomogramme de Brenner et Staebelin[22]. Dans nos publications précédentes, nous indiquions les quantités de sulfate ammonique à ajouter à la solution de protéines en pourcent de la quantité de sulfate ammonique qu'il faut ajouter à 1 l d'eau pour obtenir la saturation à 0°, c'est-à-dire 706 g.

Les fractionnements par la chaleur se font à 50° pendant 15 min sur des solutions dialysées au préalable contre de l'eau distillée ou selon la méthode employée par Baranowski[15] pour l'isolement des myogènes cristallisés A et B de lapin: les protéines du filtrat obtenu après broyage et extraction du muscle 1 h par un égal volume d'eau, sont précipitées entre 45 et 65 % de saturation avec du sulfate ammonique solide (réactif pour analyse). Cette fraction, dissoute dans un faible volume d'eau, est chauffée à 52° pendant 15 min. Après centrifugation, le liquide surnageant est concentré et ajusté au pH 5.9. Additionné de faibles quantités de sulfate ammonique, il donne naissance chez le lapin à des cristaux de myogène A.

Méthodes d'analyse

1. Les électrophorèses sont réalisées à 1° au moyen d'un appareil décrit par Dubuisson, Distèche et Debot[23], différant de l'appareil de Tiselius classique par la section rectangulaire du tube en U. Le dispositif d'enregistrement est du type Longsworth[24, 25]. Les surfaces des gradients ont été mesurées suivant la méthode de Tiselius et Kabat[26]. L'effet ε, très faible aux forces ioniques utilisées, a été négligé, mais il a été tenu compte de l'effet δ[27]. L'erreur introduite par la superpositon de faibles gradients salins aux gradients protéiniques est négligeable dans nos conditions d'expériences[13]. Dans la reproduction des clichés, la partie supérieure de la figure correspond au diagramme ascendant et le sens de la migration est indiqué par la flèche.

2. Les ultracentrifugations ont été faites au Laboratoire de Chimie-Physique de l'Université de Liège (Prof. V. Desreux) au moyen d'une ultracentrifugeuse Spinco modèle E, munie du dispositif optique de Philpot[28]. La constante S_{20} est corrigée pour la température de 20° et par rapport à l'eau. Les proportions relatives des divers gradients ont été évaluées suivant la méthode de Tiselius et Kabat[26].

3. Les mesures de diffusion, réalisées également au Laboratoire de Chimie-

Physique, ont été effectuées à 4° au moyen d'une cellule à électrophorèse de Tiselius avec la méthode optique de **Longsworth**[24]. La constante de diffusion D_{20} a été calculée par la méthode de l'ordonnée maximum et corrigée par rapport à l'eau et pour la température de 20°.

4. Les spectres d'absorption dans l'u.v. ont été mesurés au moyen d'un spectrophotomètre Beckman D.U. par rapport à un blanc de même composition saline.

5. La méthode de microcataphorèse selon **Kunitz et Northrop** a été employée pour la détermination du p.i. du "myogène I" en présence de tampons acétiques de μ 0.1 et pH 4.3, 4.6, 5.01 et 5.30, de tampons cacodyliques de μ 0.1 et pH 6.0 et 5.67 et de tampons phosphatiques de μ 0.1 en phosphate et μ 0.05 en NaCl et de pH 6.15, 6.74 et 7.3. Les mesures ont été effectuées suivant le mode opératoire décrit par **Theunissen-Van Zyp**[29]. Compte tenu des résistivités légèrement différentes des divers tampons employés, et tous les autres facteurs étant constants, la vitesse de migration V, exprimée en unités arbitraires, est égale à $K \times 1/t \times \varrho_m/\varrho$, t étant le temps en secondes, ϱ_m la moyenne des résistivités des tampons et ϱ, la résistivité du tampon employé pour la détermination actuelle. K est une constante instrumentale.

6. Les dosages d'acides aminés ont été faits par G. **Duchâteau**, dans le Laboratoire de Biochimie de l'Université de Liège (Prof. M. **Florkin**), par voie microbiologique. Le détail de la technique employée a été décrit par **Duchâteau et Florkin**[30].

RÉSULTATS EXPÉRIMENTAUX

Analyse électrophorétique

La teneur en protéines de nos préparations de myogène varie quelque peu; elle est de 1.3 g pour 100 ml d'extrait chez la carpe miroir de 1 à 2 kg et chez la plie, mais atteint environ 2 % chez la carpe miroir et la carpe commune pesant 4 à 5 kg. Le mode de broyage est sans influence sur la quantité de protéines extraites.

Les électrophorèses prolongées de myogène de carpe miroir de 1 à 2 kg permettent de distinguer trois groupes de gradients (Fig. 1A, Tableaux I et II): un groupe rapide I homogène, un groupe II de vitesse moyenne et composé de trois gradients dont le principal correspond à 40 % du myogène total, et un groupe III, plus lent, qui se sépare en deux composants d'ailleurs mal définis. Les expériences réalisées à partir de carpes communes ou de carpes miroirs âgées (pesant de 4 à 5 kg) révèlent les mêmes

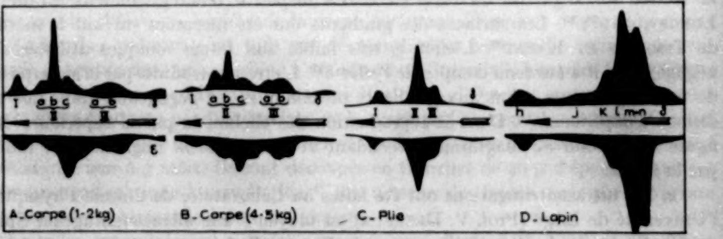

Fig. 1. Diagrammes électrophorétiques de myogènes à μ 0.15 et pH 7.3. (a) Carpe miroir de 1.5 kg (champ: 4.32 V/cm; durée: 435 min; concentration: 2.1 %); (b) carpe commune de 5 kg (champ: 4.81 V/cm; durée: 418 min; concentration: 1.87 %); (c) plie (champ: 4.22 V/cm; durée: 322 min; concentration: 1.26 %); (d) lapin (champ: 4.06 V/cm; durée: 378 min; concentration: 2.7 %). Les lettres désignant les gradients sont celles de la nomenclature de **Jacob**.

TABLEAU I

MOBILITÉS MOYENNES DES GRADIENTS OBSERVÉS À L'ÉLECTROPHORÈSE DES MYOGÈNES DE CARPE ET DE PLIE ET DE DIVERSES FRACTIONS DE MYOGÈNE DE CARPE À μ 0.15 ET pH 7.3

A = mobilités ascendantes; D = mobilités descendantes.

Nature de l'extrait	*Nombre d'expériences*	*Concn. en protéines en %*		*Mobilités électrophorétiques moyennes × 10^5 cm²/V/sec*						
				I	*II*			*III*		
					a	*b*	*c*	*a*	*b*	*c*
Carpes (1 à 2 kg)	4	1.3 ± 0.10	A	4.41 ± 0.32	3.67 ± 0.25	3.14 ± 0.24	2.62 ± 0.30	1.91 ± 0.15	1.39 ± 0.13	—
			D	4.30 ± 0.28	3.58 ± 0.03	2.81 ± 0.18	2.20 ± 0.23	1.63 ± 0.05	1.16 ± 0.11	—
Carpes (4 à 5 kg)	3	2 ± 0.17	A	4.24 ± 0.23	3.52 ± 0.14	3.10 ± 0.14	2.56 ± 0.16	1.85 ± 0.15	1.36 ± 0.10	—
			D	4.17 ± 0.20	3.36 ± 0.11	2.76 ± 0.11	2.23 ± 0.08	1.51 ± 0.07	1.26 ± 0.19	0.84
Plies	4	1.28 ± 0.05	A	4.68 ± 0.37	—	2.62 ± 0.30	—	1.83 ± 0.36	—	—
			D	4.58 ± 0.29	3.04 ± 0.15	2.39 ± 0.27	—	1.70 ± 0.24	—	—
Fraction 0–55% (carpes de 1 à 2 kg)	2	1.55 ± 0.07	A	4.02 ± 0.03	—	2.90 ± 0.12	—	1.74 ± 0.06	—	—
			D	—	—	—	—	—	—	—
Fraction 48–60% (carpe de 1.5 kg)	1	1.8	A	—	—	3.01	—	—	—	—
			D	—	—	—	—	—	—	—
Fraction 48–60% (carpe de 5 kg)	1	1.8	A	—	—	2.91	2.34	1.78	—	0.99
			D	—	—	2.72	2.15	1.60	—	0.98
Fraction 55–70% (carpes de 1 à 2 kg)	2	1.45 ± 0.07	A	4.10 ± 0.02	—	2.86 ± 0.13	2.24 ± 0.11	—	—	0.87 ± 0.07
			D	4.08	—	2.59 ± 0.10	2.02 ± 0.08	—	—	0.75 ± 0.21
Fraction 70–90% (carpe de 1.5 kg)	1	1.8	A	4.27	3.42	3.10	2.65	—	1.29	—
			D	4.06	—	2.66	—	—	1.05	—

TABLEAU II

TENEURS ASCENDANTES MOYENNES DES PRINCIPAUX GRADIENTS OBSERVÉS À L'ÉLECTROPHORÈSE DES MYOGÈNES DE CARPE ET DE PLIE ET DE DIVERSES FRACTIONS DU MYOGÈNE DE CARPE À μ 0.15 ET pH 7.3

Nature de l'extrait	*Nombre d'expériences*	*Concn. en protéines en %*	*Teneurs ascendantes moyennes en %*					
			I	*II*			*III*	
				a	*b*	*c*	*a*	*b + c*
				7.5 ± 2.9	41 ± 8.6	16.5 ± 4.2		
Carpes (1 à 2 kg)	4	1.3 ± 0.10	14.5 ± 3.32		65 ± 6.4		20.5 ± 4.0	
				14.5 ± 2.5	25 ± 5	14.5 ± 3.8	13.5 ± 3.8	23.8 ± 4.0
Carpes (4 à 5 kg)	3	2 ± 0.17	7.5 ± 0.6		54 ± 5.7		37.6 ± 5.7	
Plies	4	1.28 ± 0.05	12 ± 2	—	68 ± 6.5	—	20 ± 3.2	—
Fraction 0–55% (carpes de 1 à 2 kg)	2	1.55 ± 0.07	—	—	56.5 ± 4.9	—	15.5 ± 3.5	—
Fraction 48–60% (carpe de 1.5 kg)	1	1.8	—	—	58	—	—	—
Fraction 48–60% (carpe de 5 kg)	1	1.8	—	—	29	16	11	44
Fraction 55–70% (carpes de 1 à 2 kg)	2	1.45 ± 0.07	—	—	54.5 ± 4.9	22 ± 1.3	—	—
Fraction 70–90% (carpe de 1.5 kg)	1	1.8	18	15	35	13	—	19

gradients, mais en proportions différentes (Fig. 1B, Tableaux I et II). Le groupe III y est particulièrement important et comprend parfois un composant supplémentaire IIIc. Macroscopiquement, les muscles blancs des carpes âgées diffèrent également de ceux des petites carpes miroirs par leur teinte rosée et leur teneur élevée en graisse.

L'électrophorèse du myogène de plie montre également trois groupes de gradients comparables à ceux de la carpe en vitesse et en quantité (Fig. 1C, Tableaux I et II). Les électrophorèses faites à μ 0.05 montrent les mêmes gradients, avec une séparation légèrement meilleure.

Comparé avec les myogènes de carpe et de plie, celui du lapin[9,10] se révèle beaucoup plus lent á l'électrophorèse: le gradient IIIb de la carpe a une mobilité encore plus élevée que le plus rapide des composants importants du myogène de lapin, le gradient I de vitesse ascendante — $1.2 \cdot 10^{-5}$ cm^2/V/sec, à μ 0.15, au pH 7.3. Le gradient IIb, si considérable chez la carpe et la plie, n'a pas d'homologue chez le lapin (Fig. 1D).

Fractionnements

Fractionnement par le sulfate ammonique à pH 7: Chez la petite carpe miroir, le myogène commence à précipiter à partir de 40 % de saturation en sulfate ammonique. Les quatre zones principales de précipitation étudiées (de 40 à 55 %, de 55 à 70 %, de 70 à 90 % et de 90 à 100 % de saturation) contiennent respectivement 7 %, 8 %, 80 % et 5 % des albumines (moyenne de deux expériences). La première montre à l'électrophorèse (μ 0.15, pH 7.3) les gradients IIb et IIIa; la deuxième, les gradients IIb et IIc; la troisième, tous les gradients du myogène à l'exception de IIIa; IIb et IIc y sont en plus faibles proportions que dans le myogène total (Fig. 2, Tableaux I et II). La quatrième fraction montre des gradients mal définis, de mobilités voisines de IIa et IIb. Ajoutons que la fraction précipitée entre 48 et 60 % de saturation est constituée principalement par le gradient IIb chez la petite carpe miroir. Elle contient aussi des protéines du gradient III chez la grosse carpe commune (Fig. 3).

En conséquence, chez la petite carpe miroir, les protéines du gradient IIIa

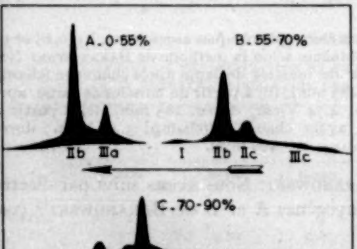

Fig. 2. Diagrammes électrophorétiques ascendants de fractions de myogène de carpe à μ 0.15 et pH 7.3. (a) Fraction 0–55 % (champ: 3.7 V/cm; durée: 291 min; concentration: 1.6 %); (b) fraction 55–70 % (champ: 3.6 V/cm; durée: 528 min; concentration: 1.4 %); (c) fraction 70–90 % (champ: 4.15 V/cm; durée: 520 min; concentration: 1.8 %).

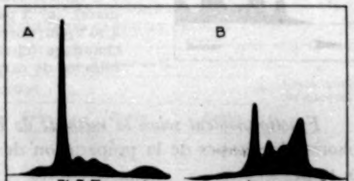

Fig. 3. Diagrammes électrophorétiques ascendants de la fraction 48–60 % de myogène de carpe à μ 0.15 et pH 7.3 (concentration en protéines: 1.8 %); (a) à partir d'une carpe miroir de 1.5 kg (champ: 4.3 V/cm; durée: 285 min); (b) à partir d'une carpe commune de 5 kg (champ: 3.54 V/cm; durée: 381 min).

précipitent donc surtout entre 40 et 48 % de saturation, celles du gradient IIb à partir de 40 %, celles du gradient IIc à partir de 55 % et celles des gradients I, IIa et IIIb seulement au dessus de 70 %.

Chez la plie, nous n'avons pas fait de fractionnements systématiques. La fraction 48–60 %, seule étudiée par électrophorèse, révèle, comme chez la carpe, un seul gradient important, le gradient IIb.

Chez le lapin, la majorité des protéines constitutives des gradients les plus abondants (l et m) précipiteraient entre 40 et 70 % de saturation, c'est-à-dire pour des valeurs moindres de concentration en sulfate ammonique que chez la carpe[31].

Thermolabilité: Le chauffage à 50° au bain-marie pendant 15 min du myogène de carpe coagule la moitié des protéines. Le liquide surnageant révèle à l'électrophorèse (Fig. 4A et B) la disparition totale du gradient IIb et partielle du groupe III. Chez le lapin, seulement le tiers des albumines est thermolabile dans ces conditions. Les gradients l et m diminuent, mais aucun n'est complètement éliminé (Fig. 4C et D).

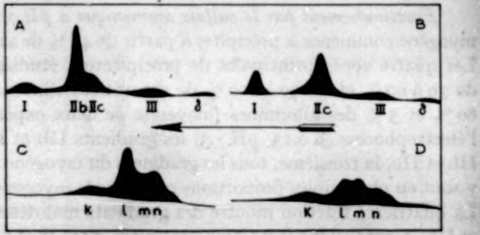

Fig. 4. Diagrammes électrophorétiques ascendants de myogènes à μ 0.15 et pH 7.3. (a) Myogène de carpe (concentration en protéines: 1.4%); (b) même extrait après chauffage à 50° et concentration du liquide surnageant (concentration en protéines: 1.26%); (c) myogène de lapin (concentration en protéines: 2%); (d) même extrait après chauffage à 50° (concentration en protéines: 1.3%).

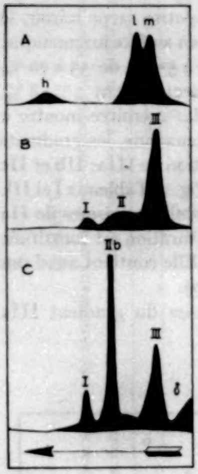

Fig. 5. Diagrammes électrophorétiques ascendants, à μ 0.15 et pH 7.3, de fractions obtenues selon la méthode de Baranowski (voir texte). (a) À partir de muscles de lapin après chauffage (champ: 4.22 V/cm; durée: 285 min); (b) à partir de muscles de carpe, après chauffage (champ: 4.74 V/cm; durée: 285 min); (c) à partir de muscles de carpe, avant chauffage (champ: 3.45 V/cm; durée: 408 min).

Fractionnement selon la méthode de Baranowski: Nous avons suivi par électrophorèse les étapes de la préparation des myogènes A et B de Baranowski[15] (voir p. 105).

Chez la carpe, la fraction des protéines précipitée entre 45 et 65 % de saturation en sulfate ammonique montre l'existence du gradient IIb et du groupe lent III (Fig. 5C). Après chauffage à 52° et élimination du précipité, la solution surnageante révèle la disparition complète du gradient IIb et l'existence prédominante du groupe III, de vitesse ascendante $-1.08 \cdot 10^{-5}$ cm^2/V/sec à μ 0.15 et pH 7.3 (Fig. 5B). Cette solution ne donne naissance qu'à une très petite quantité de cristaux en aiguilles ou en plaquettes.

Chez le lapin, la solution obtenue après chauffage est beaucoup plus riche en protéines que chez la carpe et révèle à l'électrophorèse deux gradients (Fig. 5A) de vitesses ascendantes respectivement égales à —1.2 et —$0.8 \cdot 10^{-5}$ cm²/V/sec à μ 0.15 et pH 7.3 (gradients l et m de JACOB[9, 10]). C'est à partir de cette solution que BARANOWSKI[15] obtient de nombreux cristaux de myogène A, puis de myogène B après élimination des premiers. Rien de semblable n'a été observé à partir des muscles de carpe malgré la similitude électrophorétique des solutions obtenues après chauffage (Fig. 5A et B). La méthode de BARANOWSKI détermine donc la disparition partielle des différences électrophorétiques des solutions initiales par élimination du gradient IIb. Elle ne permet néanmoins pas d'obtenir chez la carpe la cristallisation abondante observée chez le lapin.

Ultracentrifugation

Chez la carpe et la plie, trois ultracentrifugations de myogènes donnent des résultats concordants et comparables à ceux déjà obtenus par HAMOIR[16] à partir de la carpe (Fig. 6, Tableau III). Elles montrent l'existence de trois gradients bien définis.

Les fractions 48–60 % et 90–100 % du myogène de carpe ont été également

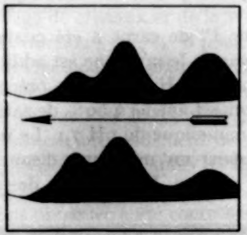

Fig. 6. Diagrammes d'ultracentrifugations de myogène à μ 0.15 et pH 7.3. En haut: myogène de carpe après 64 min à 60,000 tours/min (concentration: 1.8%). En bas: myogène de plie, après 64 min à 58,000 tours/min (concentration: 1.33%).

TABLEAU III

VITESSES DE SÉDIMENTATION ET PROPORTIONS DES GRADIENTS OBSERVÉS À L'ULTRACENTRIFUGATION DES MYOGÈNES DE CARPE ET DE PLIE

Experiences No.	*$S_{20} \times 10^{13}$*			*Teneurs en %*			*Concn. totale en g/100 ml*
	Type du gradient						
	Lent	*Moyen*	*Rapide*	*Lent*	*Moyen*	*Rapide*	
Carpe							
1*	1.70	4.85	6.86	57	36	7	1.07
2*	1.48	4.75	7.12	48	39	13	1.1
3	1.35	4.96	7.33	40	42	18	1.8
Moyennes	1.45	4.85	7.10	48	39	13	
Plie							
1	1.4	5.4	6.9	22	52	26	1.33
2	1.14	4.9	7.28	28	45	27	1.26
Moyennes	1.27	5.15	7.09	25	48.5	26.5	

* D'après HAMOIR[13].

étudiées par cette méthode; la première montre un gradient principal (60 %) de $S_{20} = 5$ S et la seconde, un seul gradient de $S_{20} = 1.45$ S. La fraction 48–60 % du myogène de plie révèle par contre deux gradients de $S_{20} = 7$ S (75 %) et 5 S (25 %).

Chez le lapin, seulement deux gradients importants de $S_{20} = 7.69$ S et 5.52 S sont visibles à l'ultracentrifugation d'extraits musculaires de force ionique finale 0.13, faite avec un tampon phosphatique de pH 5.9[18]. Les protéines de la fraction 90–100 % de la carpe n'ont donc pas de correspondant chez le lapin.

Isolement et propriétés d'albumines cristallisées

L'isolement d'albumines cristallisées de carpe et de plie a été tenté à partir des fractions 48–60 % et 90–100 %, qui n'ont pas d'homologues chez le lapin. L'une est formée des protéines du gradient électrophorétique IIb, l'autre, par celles du gradient de constante de sédimentation de 1.45 S.

On a groupé sous le nom de "myogène I" et "myogène II", les protéines cristallisées à partir de la première et de la seconde fraction, respectivement.

Le "myogène I" de carpe

Isolement: Le "myogène I" de carpe a été cristallisé à partir de la fraction 48–60 % par la méthode suivante: le myogène est additionné de sulfate ammonique solide jusqu'à 48 % de saturation (pH 5.8 à 6), le précipité est éliminé par centrifugation et le liquide surnageant est amené à 60 % de saturation par adjonction d'une solution saturée de sulfate ammonique de pH 7.1. Le nouveau précipité, séparé par centrifugation, est dissous dans un minimum d'eau. La solution ainsi obtenue (solution A) a un pH de 6.5 environ. L'adjonction de sulfate ammonique en petites

Fig. 7. Microphotographie en contraste de phase (gross. 1,160 ×) de cristaux de "myogène I" formés à pH 6.5.

quantités y provoque la formation de petits cristaux en aiguilles isolés ou groupés en faisceaux (Fig. 7). Cette protéine cristallisée est la première obtenue à partir de muscles de poisson[32]. A pH 7, on obtient aussi parfois des cristaux identiques de formation plus lente et qui grossissent pendant plusieurs mois. Il est également possible d'en obtenir à partir des fractions 40–55 et 55–70 %. Leur formation est toutefois généralement accompagnée de précipité amorphe.

Cette méthode d'isolement n'est valable telle quelle qu'à partir de muscles de petites carpes miroirs. Avec les grosses carpes communes ou miroirs, en effet, la fraction 48–60 % qui montre à l'électrophorèse deux gradients principaux (Fig. 3) donne toujours naissance à un abondant précipité amorphe conjointement à l'apparition de cristaux. Le précipité amorphe peut être éliminé en divisant la fraction 48–60 % en deux sous-fractions: à l'électrophorèse, la supérieure (55–60 %) est formée presqu' uniquement par le gradient IIb et elle donne naissance uniquement aux cristaux de "myogène I", l'inférieure (48–55 %) contient surtout des protéines du groupe électrophorétique III (précipité amorphe). Les cristaux sont isolés par filtration sur plaque poreuse et lavés rapidement avec une solution de sulfate ammonique à 40 %.

Le rendement de la cristallisation est d'environ 1 % du myogène. Si l'on tient compte des pertes dues au lavage de cristaux et de la possibilité d'obtenir également du "myogène I" à partir de la fraction 55–70 %, il pourrait être estimé à 4 %. Etant donné le mauvais rendement des méthodes de cristallisation, le "myogène I" constitue probablement une proportion de myogène total supérieure à 4 % et égale au moins à celle des gradients IIb dans les fractions 40–55 et 55–70 % (soit 9 % des albumines musculaires). BARANOWSKI[15] obtient un rendement de 10 % en ce qui concerne les cristaux de myogène A du lapin mais, même après lavage et recristallisation, ceux-ci donnent des solutions hétérogènes à l'électrophorèse[19, 31].

La pureté des préparations obtenues a été contrôlée par électrophorèse et ultracentrifugation. Les cristaux redissous dans le tampon phosphatique de μ 0.15 et pH 7.3, donnent des solutions homogènes par ces deux méthodes. Le "myogène I" migre au niveau du gradient électrophorétique IIb et a une vitesse de sédimentation de 5 S.

Propriétés: L'incrément spécifique d'indice de réfraction a été mesuré par la détermination du poids sec des protéines d'une solution d'indice de réfraction connu[33]. Il est de $185.5 \cdot 10^{-6}$ à 25° et 580 mμ (moyenne de trois déterminations), valeur très proche de celle obtenue par HAMOIR[13] pour le myogène total de carpe.

Le point isoélectrique, mesuré par la méthode de microcataphorèse, a été trouvé égal à 5.51, 5.50 et 5.65 lors de trois déterminations (moyenne = 5.55). Il n'est pas influencé par la présence de phosphates; les points obtenus aux pH les plus bas (tampons acétiques et cacodyliques) se situent sur la courbe obtenue au moyen de tampons phosphatiques. Cette valeur du p.i. est nettement inférieure à celle proposée par ROTH[34] qui suggère, pour le p.i. du myogène de carpe, une valeur inférieure de 0.2 à 0.3 unités pH seulement à celle du p.i. moyen du myogène de lapin (6.3 d'après WEBER[35]). D'autre part, la pente des courbes de mobilité électrophorétique en fonction du pH est plus forte chez la carpe que chez le lapin: les protéines migrant à la vitesse de $-3 \cdot 10^{-5}$ cm^2/V/sec à pH 7.3 et μ 0.15 dans le myogène de lapin ont un point isoélectrique de 5 environ (cas du gradient j de JACOB[9, 10]) et non de 5.5 comme chez la carpe.

Le poids moléculaire a été calculé au moyen de la formule habituelle, M =

$(RT\,S_{20}/D_{20}(1-V\varrho))$, où R est la constante des gaz, T, la température absolue correspondant à 20° et ϱ, la densité de l'eau à 20°. S_{20}, la constante de sédimentation extrapolée pour la concentration O, est égale à $5\cdot10^{-13}$ (Tableau IV). Elle ne varie d'ailleurs pas en fonction de la concentration en protéines dans nos conditions d'expérience. D_{20}, la constante de diffusion, est égale à $6.97\cdot10^{-7}$ (Tableau IV). En ce qui concerne le volume partiel spécifique V, nous avons utilisé la valeur de 0.745 donnée par SVEDBERG ET PEDERSEN[36] à 20°. Le poids moléculaire obtenu est de 67,000[37].

TABLE IV

VITESSES DE SÉDIMENTATION ET CONSTANTES DE DIFFUSION DU "MYOGÈNE I"

Expérience No.	*Tampon*		*Concn. en protéine en g/100 ml*	$S_{20}\times10^{13}$
	μ	*pH*		
1755	0.15	7.3	0.6	4.96
1855	0.15	7.3	1	4.90
1108	0.10	7.6	0.37	5.00
1110	0.10	7.6	0.76	4.74
1748	0.15	6.42	0.2	4.96
1076	0.10	7.6	0.49	5.00
				$D_{20}\times10^{7}$
26	0.15	7.3	0.60	7.04
28	0.15	7.3	0.36	6.89
30	0.15	7.3	0.38	6.99
			Moyenne:	6.97

Le rapport de friction f/f_0, calculé de la façon habituelle, est égal à 1.124. La molécule est donc peu asymétrique ainsi qu'il en est généralement pour les protéines du myogène. Le rapport des axes a/b, calculé à partir de tables dressées au moyen d'une formule de HERZOG, ILLIG et KUDAR et de PERRIN[36] est égal à 3.

Le spectre d'absorption dans l'u.v. du "myogène I" à pH 7.3, présente l'aspect habituel. Il passe par un minimum à 251 mμ et par un maximum à 280 mμ. Les densités optiques correspondantes rapportées à une concentration de 1 % et une épaisseur de 1 cm sont de 4.12 et 9.38 (moyenne de 5 déterminations). Le filtrat perchlorique[38, 39] de la solution de "myogène I" ne présente pas de bande d'absorption.

La teneur en acides aminés du "myogène I" a été comparée à celles de diverses protéines du myogène et à une myosine (Tableau V). Aucune ressemblance significative caractéristique des albumines musculaires n'a été mise en évidence.

D'autre part, aucune relation ne semble exister entre la composition du "myogène I" et celle du pool des acides aminés libres du muscle de carpe, très riche en glycocolle (34 à 40 % du total des acides aminés) et en histidine (20 %) et riche en alanine et méthionine (5 à 10 %)[30]. Une telle absence de corrélation entre la composition du pool des acides aminés libres et celle des protéines musculaires a déjà été signalée et discutée par FLORKIN[44].

L'activité enzymatique du myogène I reste indéterminée. Il ne possède aucune activité aldolasique (test de HERBERT, GORDON, SUBRAHMANYAN ET GREEN[45]) ou glyceraldehyde-phosphate-déshydrogénasique (test de CORI, SLEIN ET CORI[46]).

TABLEAU V

COMPOSITION EN ACIDES AMINÉS DE DIFFÉRENTES PROTÉINES DU MYOGÈNE ET D'UNE MYOSINE*

	Myogène I (carpe)	*Myogène total** (lapin)*	*Aldolase*** (lapin)*	*Glycéraldéhyde phosphate deshydrogénase§ (lapin)*	*Myosine§§ (lapin)*
Alanine	2.43		8.56	7.3	6.5
Arginine	6.38		6.33	5.2	7.36
Ac. aspartique	*12.34*		*9.7*	*10.6*	*8.9*
Ac. glutamique	*12.82*		*11.40*	5.7	*22.1*
Glycocolle	8.14		5.61	5.9	1.9
Histidine	4.07		4.21	5	3.41
Isoleucine	6.92		7.9		15.6
Leucine	*9.81*		*11.5*		
Méthionine	3.21	2.8	1.17	2.7	3.4
Lysine	*10.16*		9.54	9.7	*11.92*
Phénylalanine	5.58		3.06	5.8	4.3
Proline	4.36		5.71	3.4	1.9
Thréonine	5.61		7.47	5.2	5.1
Tyrosine	3.43	4.21	5.31	4.6	3.4
Valine	6.7		7.4	9.2	2.6
Cystine		1.96	2.24		2.8
Tryptophane		1.51	2.31	2.1	0.8
Sérine			7.3	4.4	4.33
Total	101.96		115.6		

[*] En g/100 g. Les teneurs des acides aminés les plus abondants (> 9 %) sont en italiques.
[**] BAILEY[40].
[***] VELICK ET RONZONI[41].
[§] VELICK ET UDENFRIEND[42].
[§§] BAILEY[43].

TABLEAU VI

Fractions	*Quantité totale de protéines précipitées**	*Concn. en protéine en g/100 ml***	*Gradients observés à l'U.C. $S_{20} \times 10^{13}$*	*Teneur en % des gradients observés à l'U.C.*	*pH de cristallisation*	*Aspect des cristaux*
Fractions 48–60%						
No. 1	**6%**	**1.0**	**7.4***** **5**	**85** **15**	**6.5**	**Précipité amorphe,**
No. 2	**5%**	**0.6**	**7.3***** **5.9**	**70** **30**		**Rares cristaux rhomboédriques**
Fraction 48–55%	**1.2%**	**0.8**	**7.13**	**100**	**7**	**Cristaux en aiguilles**
Fraction 55–60%	**3.8%**	**0.8**	**7.25** **5.33**	**55** **45**	**6.5**	**Cristaux rhomboédriques**

[*] Quantité de protéines de la fraction en g/100 g de protéines du myogène total.
[**] Concentration des solutions soumises à l'ultracentrifugation.
[***] Mesures imprécises par suite de l'étalement du gradient.

Les "myogènes I" de plie

La méthode utilisée pour la cristallisation du "myogène I" à partir de la fraction 48–60 % du myogène de carpe, ne permit d'obtenir, chez la plie, que quelques rares cristaux avec un précipité amorphe abondant. La fraction 48–60 % du myogène de plie, presqu'homogène à l'électrophorèse, est, en effet, hétérogène à l'ultracentrifugation, contrairement à ce qui avait été observé avec la carpe, et comporte deux constituants importants de $S_{20} = 7\,S$ (75 %) et 5 S (25 %). Le premier peut être isolé entre 48 et 55 % de saturation. Entre 55 et 60 %, le précipité obtenu révèle toujours deux constituants à l'ultracentrifugation mais en proportions presqu'égales (Tableau VI).

Les fractions 48–55 % et 55–60 % sont obtenues respectivement aux pH 6.3 et 6.5 par saturation jusqu'à 48 % avec du sulfate ammonique solide, ensuite avec une solution saturée de pH 7.1.

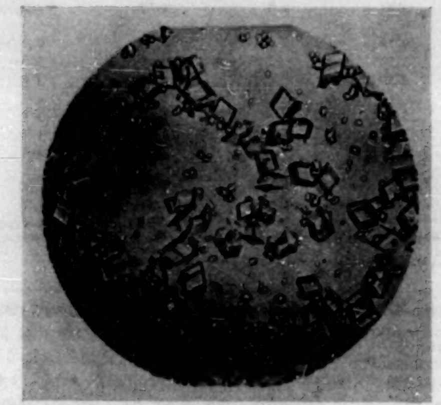

Fig. 8. Microphotographie de cristaux de "myogène Ib" de plie formés à pH 6.5 (gross.: 210 ×).

La fraction 48–55 % mise à cristalliser, c'est-à-dire dissoute dans un minimum d'eau avec adjonction subséquente de sulfate ammonique, donne lieu à la formation d'un précipité amorphe, à pH 6.3. A pH 7, par contre (après reprécipitation de la protéine avec une solution saturée de sulfate ammonique ajustée à ce pH et redissolution subséquente du précipité), la formation d'un grand nombre de cristaux en aiguilles semblables à ceux du "myogène I" est observée (en collaboration avec JEBSEN, expériences non publiées).

La fraction 55–60 % donne naissance, au pH 6.5, à de nombreux et gros cristaux rhomboédriques (Fig. 8), mêlés à un léger précipité amorphe facilement éliminé par lavage[47]. Ces cristaux peuvent également être obtenus à partir de la fraction 60–70 %, mais en plus faible quantité.

Chez la plie, la fraction 48–60 % contient donc au moins deux constituants importants. Ils migrent à l'électrophorèse au niveau du gradient IIb. Le premier, appelé "myogène Ia", précipite dans les zones inférieures de saturation en sulfate ammonique et a un S_{20} de 7.2 S; le second, le "myogène Ib", précipite dans les zones supérieures et a un S_{20} de 5.3 S (et non 7.3 comme il a été précédemment publié par erreur)[47].

Le "myogène II" de carpe

Isolement: Le précipité obtenu entre 90 et 100% de saturation et à pH 6.5 est blanc, dépourvu de myoglobine, et requiert 1 h pour se former complètement. Il constitue 5% du myogène total; il est toutefois plus abondant dans les extraits musculaires de grosses carpes communes. Dissous dans un minimum d'eau et mis à cristalliser, il donne naissance à de nombreux cristaux souvent visibles à l'oeil nu (Fig. 9). La protéine ainsi cristallisée a été appelée "myogène II"[48].

Fig. 9. Microphotographie en contraste de phase de cristaux de "myogène II" (gross.: 450 ×).

Electrophorèse et ultracentrifugation: Le "myogène II" montre à l'électrophorèse (μ 0.15, pH 7.3) deux gradients voisins, mal définis, dont le plus important migre au niveau du gradient IIa du myogène total. Une préparation électrophorétiquement pure de "myogène II" a été récemment obtenue par la méthode d'électrophorèse de zone[49]. Notons que le gradient IIa se trouve en plus grande proportion chez la carpe commune, ce qui concorde avec l'abondance plus grande de "myogène II" dans les muscles de celle-ci. A l'ultracentrifugation, le "myogène II" est homogène. Sa constante de sédimentation S_{20} est de 1.44 S dans des solutions à 0.4% en protéines.

Spectre d'absorption dans l'u.v. Le spectre d'absorption dans l'u.v. des solutions de "myogène II", à pH 7.3, diffère nettement du spectre habituel des protéines (Fig. 10). Il présente trois maxima à 253, 259 et 264 mμ, identiques à ceux de la phénylalanine à pH neutre[50], et une faible absorption à 280 mμ, probablement due au tryptophane ou à la tyrosine.

Le "myogène II" a donc une teneur élevée en phénylalanine; elle a été estimée spectrophotométriquement par HAMOIR[49] à 15.5 % et est égale à 14 % d'après l'analyse d'acides aminés faite par KOMINZ (communication personnelle). Par contre, il contient très peu de tryptophane et de tyrosine. Il ne contient pas de nucléotides; la réaction à l'orcinol sur le filtrat perchlorique est négative et son spectre dans l'u.v. ne présente pas d'absorption à 260 mμ.

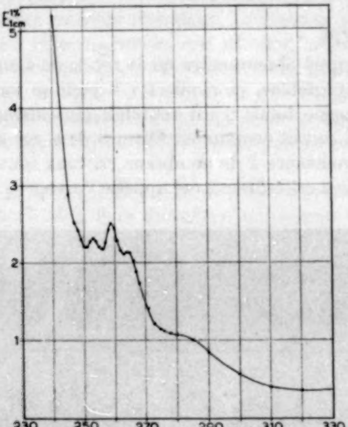

Fig. 10. Spectre d'absorption dans l'u.v. d'une solution de "myogène II" à pH 7.3. En ordonnée: la densité optique d'une solution contenant 1 % de protéines sous une épaisseur de 1 cm; en abscisse: la longueur d'onde en mμ.

Certaines polymyxines[51] et la gélatine qui contient 2 % de phenylalanine et pas de tryptophane ni de tyrosine[52] sont les seuls protides jusqu'ici décrits, possédant une composition semblable en acides aminés[53].

Un composé semblable au "myogène II" existe également chez la plie[54].

DISCUSSION ET CONCLUSION

1. Les extraits musculaires à faible force ionique de divers téléostéens présentent d'une façon constante une mobilité électrophorétique moyenne élevée d'environ —3 cm^2/V/sec, à μ 0.15 et pH 7.3[1,13,14,55]. L'étude du myogène de morue[55] suggérait que les albumines musculaires devaient se comporter de façon semblable. Nos recherches sur les myogènes de carpe et de plie ont confirmé ce résultat. Elles montrent, de plus, l'existence de trois groupes d'albumines bien définis qui se retrouvent, à quelques différences près, dans le myogène de morue. Ils ne s'observent pas dans les extraits à faible force ionique dont les diagrammes sont très variables suivant l'espèce[14]. L'élimination de la globuline X permettrait donc de dégager certaines régularités. Comme chez la morue, des variations quantitatives des gradients électrophorétiques en fonction de l'âge ont été observées dans le myogène de carpe. Notons

également une solubilité élevée des albumines musculaires de carpe en présence de sulfate ammonique et une thermolabilité considérable à 50°. Ces mêmes propriétés se retrouvent aussi chez la plie[54]. Enfin, à l'ultracentrifugation, les myogènes de carpe et de plie se répartissent en trois groupes de constante de sédimentation égale à 1.5, 5 et 7.3 S, également observés dans les extraits à faible force ionique de muscles de morue[56].

Par contre, chez le lapin, la mobilité électrophorétique moyenne du myogène calculée à partir des recherches de JACOB[57], est peu élevée (environ 1 $cm^2/V/sec$ à μ 0.15 et pH 7.3). La thermolabilité et la solubilité en présence de sulfate ammonique sont moins grandes que chez la carpe et les ultracentrifugations d'extraits musculaires à faible force ionique montrent l'absence d'un gradient lent de 1.5 S[18].

La comparaison de nos résultats avec ceux de la littérature permet donc de dégager l'existence d'une similitude marquée entre les myogènes de poissons et d'une différence considérable entre ceux-ci et le myogène de lapin.

2. Deux groupes de protéines sont principalement responsables de cette différence et pourraient être considérés comme caractéristiques des téléostéens. Le premier est formé des protéines de mobilité électrophorétique d'environ —3 $cm^2/V/sec$, à μ 0.15 et pH 7.3; elles constituent respectivement 40 à 60 % des myogènes de carpe et de plie, sont thermolabiles à 50° et précipitées par le sulfate ammonique surtout dans la fraction 48 à 60 % de saturation. Le second constitue la composante lente des ultracentrifugations de myogène de poisson ($S_{20} = 1.5$ S); il est le seul à précipiter dans la fraction 90 à 100 % de saturation en sulfate ammonique.

3. Le "myogène I" de carpe et les "myogènes Ia et b" de plie ont été cristallisés à partir du premier groupe.

Le p.i. du "myogène I" qui par son abondance et sa situation dans les diagrammes représente le comportement électrophorétique moyen du myogène de carpe, est de 5.5. Il est du même ordre de grandeur chez la morue[55, 58] alors qu'il est de 6.5 pour la moyenne des albumines de lapin[9, 10]. Cet écart entre le p.i., de même que l'allure différente des courbes de mobilités en fonction du pH, doivent entraîner des différences dans le tamponnement des muscles de poissons et de lapin, qui n'ont pas encore été étudiés. Ces données sont par ailleurs en accord avec celles de RENARD[5] qui observe une diminution progressive de la mobilité moyenne des myogènes, à pH 7, quand on monte dans l'échelle des vertébrés. D'autre part, la détermination du P.M. du "myogène I", qui est inférieur de moitié à celui des myogènes A et B du lapin[16, 17] a permis, pour la première fois, de préciser les différences importantes présentées à ce point de vue par les myogènes, suivant leur origine animale.

Le "myogène Ib" de la plie possède des propriétés analogues au précédent, bien que la forme de ses cristaux soit différente. Par contre, le "myogène Ia", dont la vitesse de sédimentation est beaucoup plus élevée, devrait sans doute être considéré comme une protéine d'une autre catégorie.

Quant au "myogène II", cristallisé à partir du second groupe de protéine, son spectre dans l'u.v. révèle une composition en acides aminés cycliques très particulière, qui n'a jamais été signalée à propos d'autres classes animales, et sa masse moléculaire est vraisemblablement très faible. Un composé semblable existe également chez la plie[54]. L'abondance de petites molécules, absentes du myogène de lapin, a été également signalée chez la grenouille[18]; elle pourrait être caractéristique des muscles des animaux à sang froid.

4. La signification des différences observées entre les myogènes de poisson et de lapin n'est pas connue. Elles pourraient correspondre à la présence de protéines spécifiques d'une classe animale, ce qui est peu probable si les myogènes sont des mélanges d'enzymes essentiels au fonctionnement des muscles de tous les vertébrés[4, 36]. Elles pourraient aussi impliquer que des fonctions enzymatiques semblables peuvent être portées par des molécules par ailleurs très différentes. Les seules recherches faites dans ce sens sont celles de VELICK et coll.[59] sur les glycéraldéhyde phosphate déshydrogénases de levure et de lapin; celles-ci ne diffèrent que par leurs propriétés électrocinétiques, leur stabilité à pH acide et leur pouvoir antigénique. Notre travail rend vraisemblable l'existence de différences beaucoup plus considérables entre les propriétés de certains enzymes homologues, suivant leur origine animale.

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Nous tenons à exprimer notre gratitude à Monsieur le Professeur DUBUISSON de l'attention avec laquelle il a suivi nos travaux et de l'aide qu'il nous a apportée. Nous remercions aussi très sincèrement Monsieur G. HAMOIR, agrégé, pour ses nombreux et judicieux conseils. Notre reconnaissance va également à Madame G. DUCHÂTEAU, à M. M. les Professeurs DESREUX et FLORKIN de l'Université de Liège et à M. LELOUP, Directeur de l'Institut d'Etudes maritimes d'Ostende. Enfin, nous remercions tout particulièrement le personnel du Laboratoire de Biologie générale de son assistance aussi précieuse qu'agréable.

RÉSUMÉ

La comparaison des myogènes de carpe, de plie et de lapin par les méthodes d'électrophorèse, d'ultracentrifugation et de fractionnement permet de dégager l'existence d'une similitude marquée entre les extraits homologues de poisson et d'une différence considérable entre ceux-ci et le myogène de lapin. Les myogènes de poisson possèdent une mobilité électrophorétique plus élevée, une thermolabilité et une solubilité en présence de sulfate ammonique plus grande et une vitesse de sédimentation moyenne plus faible que les albumines musculaires de lapin.

Le groupe des protéines constitutives du gradient électrophorétique principal et le groupe des protéines de faible vitesse de sédimentation semblent particulièrement caractéristiques des myogènes de poisson par rapport à celui de lapin.

Une albumine de carpe et deux albumines de plie, appelées "myogènes I", ont été isolées et cristallisées à partir du premier groupe. Elles se distinguent par leur mobilité électrophorétique élevée et leur abondance dans le myogène. Une albumine de carpe, appelée "myogène II" a été isolée à partir du second groupe. Elle est caractérisée par son P.M. très faible et sa composition en acides aminés cycliques particulière. L'absence de protéines semblables dans le myogène de lapin met en évidence l'existence de différences considérables entre les propriétés des albumines musculaires, par ailleurs douées des mêmes activités enzymatiques, suivant leur origine animale.

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MÉTHODE DE MESURE DES PRINCIPALES VOIES DU MÉTABOLISME CALCIQUE CHEZ L'HOMME

JEAN-PAUL AUBERT ET GERARD MILHAUD

Laboratoire des Isotopes, Institut Pasteur, Paris (France)

(Reçu le 3 juin, 1959)

SUMMARY

A method of measuring the principal routes of calcium metabolism in man

The method is based on the following techniques and theory: (1) Intravenous injection of a negligible weight of $^{45}CaCl_2$. (2) Determination of the specific activity of the serum, of the total activity in urine and stool and of the amount of ingested and excreted calcium. (3) Mathematical analysis of the curve showing the decrease of the specific activity of the serum calcium.

Thus data are obtained about (a) the intestinal absorption of calcium present in food. (b) The quantity of calcium excreted by the intestinal tract, differentiated from unabsorbed calcium. (c) The rate of the physiological renewal processes of the skeleton through osteolysis and osteoblastosis. (d) The amount of readily exchangeable calcium, which is the calcium pool of the organism. This pool consists of three portions, which can be identified as clearly defined physiological entities, each having a different velocity constant of exchange with serum calcium. (e) The rate of exchange of serum calcium with these different portions of the pool. (f) The quantity of bone calcium undergoing recrystallisation.

Examples are given of application of the method in man and the theory is verified in the rat. The accuracy of the described method is discussed and compared with the simplified method that is usually employed.

INTRODUCTION

Nos connaissances actuelles de la physiologie du calcium chez l'homme et celles de la physico-chimie des sels osseux permettent maintenant d'envisager une étude quantitative des différents aspects du métabolisme de cet élément.

Le calcium est présent dans tous les tissus et le sang représente un compartiment central dans lequel entre du calcium exogène et endogène et duquel sort du calcium qui est excrété ou se fixe dans d'autres parties de l'organisme.

Les grandes voies physiologiques du métabolisme calcique sont les suivantes: (a) Une partie seulement du calcium alimentaire est absorbée par la muqueuse intestinale et passe dans le sang. (b) Le calcium est excrété par le rein et l'intestin, les selles contiennent donc à la fois le calcium alimentaire non absorbé et le calcium excrété. (c) L'os, soumis aux phénomènes d'ostéolyse et d'ostéoblastose, est en continuel renouvellement.

Les études physico-chimiques des sels osseux ont montré que le calcium de ces sels s'échange avec celui des liquides ambiants et on distingue deux phénomènes.

(a) L'échange isoionique, phénomène rapide de surface, qui porterait essentiellement sur l'excès de calcium que les sels osseux renferment par rapport à la molécule de phosphate tricalcique. (b) L'échange par recristallisation, phénomène plus lent qui correspondrait à la pénétration du calcium facilement échangeable à l'intérieur de la molécule de phosphate fondamentale.

Il est impossible par les méthodes classiques de mesurer l'intensité de tous ces processus; le seul dosage chimique du calcium ne permet de connaître que la calcémie, la calciurie, le calcium ingéré et le calcium fécal. La calcémie varie relativement peu, les autres dosages donnent une image lointaine de l'ensemble du métabolisme.

L'emploi du calcium radioactif a fait apparaître l'aspect dynamique du métabolisme calcique$^{2-9}$ et le but de ce travail est de proposer une méthode qui permette de mesurer, sans aucune perturbation du système existant, l'intensité des différents processus définis ci-dessus, dans les cas normaux ou pathologiques.

PRINCIPE DE LA MÉTHODE

La mesure de l'intensité des différents processus du métabolisme calcique est fondée sur la combinaison des résultats fournis par les bilans classiques et l'interprétation théorique de la courbe de décroissance de la radioactivité spécifique du calcium sérique après injection intraveineuse de ^{45}Ca.

Analyse de la courbe de décroissance de la radioactivité spécifique du calcium sérique

La variation en fonction du temps de la radioactivité spécifique du calcium sérique après l'injection intraveineuse d'une quantité pondéralement négligeable de calcium marqué est représentée dans un système semi-logarithmique par une courbe du type de celle rapportée sur la Fig. 1.

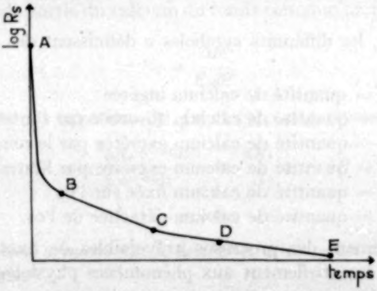

Fig. 1. Représentation de la variation du logarithme de la radioactivité spécifique du calcium sérique en fonction du temps.

La courbe peut être divisée en différents segments:

Segment AB: Ce segment correspond à une période comprise entre 0 et 48 h, il traduit une fonction mathématique assez complexe.

Segment BC: Ce segment correspond à une période comprise entre 2 et 7 jours;

le plus souvent il peut être assimilé à une droite, c'est-à-dire que dans cette période la fonction suit une loi exponentielle simple.

Segments CD et DE: Si on prolonge les mesures jusqu'à quinze jours, on s'aperçoit que la courbe tend à se redresser (segment CD) et le segment DE, qui correspond à une période comprise entre 10 et 18 jours, peut à nouveau être assimilé à une droite.

Les différents segments de la courbe permettent d'étudier séparément les processus qui nous intéressent. Le segment BC est pratiquement linéaire; la radioactivité spécifique du calcium échangeable de l'organisme est donc pratiquement homogène et seules les entrées de calcium non radioactif dans cette masse échangeable et les sorties de calcium radioactif hors de cette masse échangeable sont responsables de la diminution de la radioactivité spécifique du calcium sérique. Nous exposerons pour commencer l'interprétation de cette partie de la courbe.

Interprétation de la décroissance monoexponentielle du segment BC

Représentons schématiquement les grandes voies du métabolisme calcique (Fig. 2).

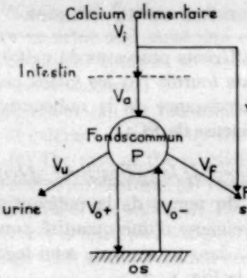

Fig. 2. Représentation schématique du métabolisme calcique.

Sur cette figure, les différents symboles v définissent des quantités de calcium par unité de temps.

v_i = quantité de calcium ingérée
v_a = quantité de calcium absorbée par l'intestin
v_u = quantité de calcium excrétée par le rein
v_f = quantité de calcium excrétée par l'intestin
v_{o+} = quantité de calcium fixée sur l'os
v_{o-} = quantité de calcium détachée de l'os.

v_{o+} et v_{o-} expriment des processus irréversibles de fixation et de départ du calcium osseux, liés essentiellement aux phénomènes physiologiques du renouvellement du squelette.

Le dosage chimique du calcium permet de mesurer le calcium ingéré v_i, le calcium urinaire v_u et le calcium total des selles F, c'est-à-dire $(v_i - v_a) + v_f$.

Entre deux et sept jours, la décroissance de la radioactivité spécifique (R_S) du calcium sanguin ou urinaire (exprimée par exemple en coups/min/mg de calcium) suit une loi exponentielle du type:

$$R_S = A\,\mathrm{e}^{-at} \tag{1}$$

Expérimentalement, A est nettement inférieur à la radioactivité spécifique initiale, définie comme le rapport entre la radioactivité totale injectée et la masse du calcium sanguin.

Cela signifie que le calcium injecté a été dilué dans une masse beaucoup plus grande de calcium, le fonds commun calcique, dont la radioactivité spécifique s'équilibre en 48 h environ avec celle du calcium sanguin.

La connaissance expérimentale de A et de a permet de calculer la vitesse globale v_T de sortie du calcium hors du fonds commun.

En toute logique $v_T = v_u + v_f + v_{o+} - v_{o-}$, mais on peut admettre sans grande cause d'erreur que, pendant le temps relativement court de l'épreuve, le calcium détaché de l'os par les processus physiologiques n'est pas radioactif. De sorte que v_T est égal en réalité à: $v_u + v_f + v_{o+}$; cette expression traduit physiologiquement la somme de l'intensité de la fixation irréversible du calcium sur l'os, du moins dans le laps de temps considéré, et de l'excrétion urinaire et fécale.

Calcul du fonds commun (P): Soit R_i la radioactivité totale injectée, l'équation (1) indique qu'au temps $t = 0$

$$R_{S_0} = A = \frac{R_i}{P} \tag{2}$$

La masse du fonds commun est donc donnée par la relation:

$$P = \frac{R_i}{A} \tag{3}$$

Calcul de la vitesse totale de sortie du calcium du fonds commun (v_T): Soit R la radioactivité totale du calcium échangeable,

$$R = PR_S \tag{4}$$

D'après les équations (3) et (1), cette expression devient:

$$R = R_i \, e^{-at} \tag{5}$$

La vitesse totale de sortie du calcium du fonds commun v_T ($v_T = v_u + v_f + v_{o+}$) est égale à

$$V_T = \frac{dR}{R_S dt} \tag{6}$$

En tenant compte des équations (5), (1) et (3), cette expression devient:

$$V_T = \frac{-aR_i \, e^{-at} dt}{A \, e^{-at} dt} = -\frac{aR_i}{A} = -aP \tag{7}$$

et en valeur absolue

$$v_T = aP \tag{8}$$

Mesure de l'excrétion urinaire v_u: L'excrétion urinaire est mesurée par le dosage classique de la calciurie.

Mesure de la vitesse d'excrétion fécale v_f: Plusieurs méthodes permettent de mesurer v_f.

1. La plus simple consiste à doser la radioactivité totale émise par l'urine R_u et par les selles R_f dans un laps de temps correspondant, tenant compte du retard de l'excrétion fécale sur l'excrétion urinaire. La radioactivité spécifique du calcium excrété dans l'urine à un instant donné est la même que celle du calcium sérique à cet instant[9]; on peut admettre qu'il en est de même pour le calcium excrété par

l'intestin. Dans ce cas, la vitesse d'excrétion fécale est donnée par la relation:

$$v_f = v_u \frac{R_f}{R_u} \tag{9}$$

Cette méthode ne nécessite pas le tracé de la courbe de décroissance et peut être utilisée avantageusement dans une étude des seules vitesses d'excrétion.

2. Quand on connaît l'équation (1) on peut calculer v_f en tenant compte des considérations suivantes:

$$\mathrm{d}R_f = v_f R_S \mathrm{d}t \tag{10}$$

soit, d'après l'équation (1)

$$\mathrm{d}R_f = v_f A \mathrm{e}^{-at} \mathrm{d}t \tag{11}$$

ce qui donne en intégrant

$$R_f = -\frac{Av_f}{a} \mathrm{e}^{-at} + K \tag{12}$$

comme au temps $t = 0$, $R_f = 0$

$$R_f = \frac{Av_f}{a}\left(1 - \mathrm{e}^{-at}\right) \tag{13}$$

$$v_f = \frac{aR_f}{A} \frac{1}{(1 - \mathrm{e}^{-at})} \tag{14}$$

Si on connaît la radioactivité totale R_f des selles formées entre les temps t_1 et t_2, on calcule donc v_f en appliquant l'équation suivante:

$$v_f = \frac{aR_f}{A}\left(\frac{1}{1 - \mathrm{e}^{-at}}\right)_{t_2}^{t_1} \tag{15}$$

3. Enfin si les selles recueillies ne portent pas sur une période où la décroissance est monoexponentielle, la méthode la plus sûre est de mesurer la surface S comprise entre la courbe de décroissance tracée en coordonnées normales et l'axe des abscisses entre les temps t_1 et t_2 et d'appliquer la relation:

$$v_f = \frac{R_f}{S} \tag{16}$$

Mesure de la vitesse de fixation sur l'os v_{o+}: Sachant que $v_T = v_u + v_f + v_{o+}$ et connaissant v_T, v_u et v_f, on en déduit v_{o+} par différence.

Mesure de l'absorption intestinale et de la vitesse d'ostéolyse: Le bilan calcique permet de déterminer le calcium alimentaire v_i et le calcium fécal total F.

Nous avons observé d'autre part que le fonds commun P ne varie pratiquement pas au cours de l'épreuve[9]. Ecrivons que la quantité de calcium qui entre dans le fonds commun est égale à celle qui en sort.

$$v_a + v_{o-} = v_T \tag{17}$$

D'autre part, le dosage du calcium fécal total F donne la relation:

$$F = v_f + v_i - v_a \tag{18}$$

Ces deux équations permettent de connaître la vitesse d'ostéolyse v_{o-} et le taux d'absorption intestinale v_a/v_i.

Décomposition du fonds commun en ses constituants

A partir de 48 h après l'injection, la radioactivité spécifique du calcium du fonds commun est homogène et tout se passe comme si les phénomènes physiologiques décrits ci-dessus portaient sur le calcium du fonds commun et non pas exclusivement sur le calcium sérique.

L'étude cinétique de l'homogénéisation du fonds commun doit fournir des renseignements sur la nature de ses constituants. Si on considère sur la Fig. 1 la portion de courbe AC, qui exprime la décroissance de la radioactivité spécifique du calcium sanguin entre les temps 0 et 7 jours, la décomposition graphique en une somme d'exponentielles montre que, pendant ce temps, la radioactivité spécifique du calcium sérique décroît selon une fonction qui est la somme de quatre exponentielles,

$$R_S = A_1 e^{-a_1 t} + A_2 e^{-a_2 t} + A_3 e^{-a_3 t} + A_4 e^{-a_4 t} \tag{19}$$

où A_1, A_2, A_3, A_4, a_1, a_2, a_3, a_4 sont des nombres positifs.

Comme l'homogénéisation dans le sang du calcium injecté se fait en quelques secondes (voir Fig. 5) cette expression mathématique signifie que tout se passe comme si le calcium sérique s'échangeait à des vitesses différentes avec le calcium de trois compartiments, dont les masses calciques seraient également différentes. La décroissance continuelle de la fonction avec le temps signifie d'autre part que du calcium radioactif sort irréversiblement du sang.

Représentons sur la Fig. 3 un tel schéma où M, M_1, M_2, M_3 sont les masses de calcium; v_1, v_2, v_3 les vitesses d'échange; v_T, v_{o-} et v_a ont la même signification que ci-dessus.

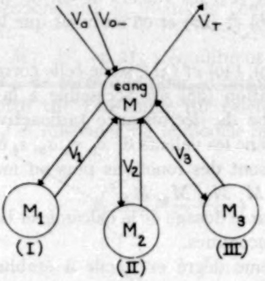

Fig. 3. Modèle de décomposition du fonds commun calcique en ses constituants.

Soit R_i la radioactivité injectée au temps zéro et R, R_1, R_2, R_3 les radioactivités totales du sang et des compartiments (I), (II) et (III) au temps t. Soit d'autre part dR, dR_1, dR_2, dR_3 les variations de la radioactivité totale dans chaque compartiment pendant le temps dt (nous considérons comme positive la radioactivité qui entre dans un compartiment et négative celle qui en sort). L'état dynamique du système s'exprime par les équations différentielles suivantes:

$$\frac{\mathrm{d}R}{\mathrm{d}t} = -\frac{v_1 + v_2 + v_3 + v_T}{M} R + \frac{v_1}{M_1} R_1 + \frac{v_2}{M_2} R_2 + \frac{v_3}{M_3} R_3 \tag{20}$$

$$\frac{\mathrm{d}R_1}{\mathrm{d}t} = \frac{v_1}{M} R - \frac{v_1}{M_1} R_1 \tag{21}$$

$$\frac{\mathrm{d}R_2}{\mathrm{d}t} = \frac{v_2}{M} R - \frac{v_2}{M_2} R_2 \tag{22}$$

$$\frac{\mathrm{d}R_3}{\mathrm{d}t} = \frac{v_3}{M} R - \frac{v_3}{M_3} R_3 \tag{23}$$

Si on résout ce système par la méthode classique en posant:

$$R = \lambda e^{st} \tag{24}$$

$$R_1 = \mu e^{st} \tag{25}$$

$$R_2 = \pi e^{st} \tag{26}$$

$$R_3 = \rho e^{st} \tag{27}$$

où
$$\lambda_i = \frac{\lambda\ (s_i)}{\mu\ (s_i)},\ \pi_i = \frac{\pi\ (s_i)}{\mu\ (s_i)},\ \rho_i = \frac{\rho\ (s_i)}{\mu\ (s_i)}$$

on doit finalement obtenir quatre intégrales du type suivant:

$$R\ = \alpha\lambda_1 e^{s_1t} + \beta\lambda_2 e^{s_2t} + \gamma\lambda_3 e^{s_3t} + \partial\lambda_4 e^{s_4t} \tag{28}$$

$$R_1 = \alpha e^{s_1t} + \beta e^{s_2t} + \gamma e^{s_3t} + \partial e^{s_4t} \tag{29}$$

$$R_2 = \alpha\pi_1 e^{s_1t} + \beta\pi_2 e^{s_2t} + \gamma\pi_3 e^{s_3t} + \partial\pi_4 e^{s_4t} \tag{30}$$

$$R_3 = \alpha\rho_1 e^{s_1t} + \beta\rho_2 e^{s_2t} + \gamma\rho_3 e^{s_3t} + \partial\rho_4 e^{s_4t} \tag{31}$$

Dans la résolution du système d'équations différentielles linéaires, s_1, s_2, s_3, s_4 sont les solutions de l'équation du quatrième degré obtenue en reportant dans les équations (20), (21), (22) et (23) les valeurs de $\mathrm{d}R/\mathrm{d}t$, $\mathrm{d}R_1/\mathrm{d}t$, $\mathrm{d}R_2/\mathrm{d}t$, $\mathrm{d}R_3/\mathrm{d}t$ tirées des équations (24), (25), (26) et (27) et en écrivant que le système est soluble (c'est-à-dire $\Delta = 0$).

Des équations (28), (29), (30) et (31), seule celle correspondant au compartiment sanguin, c'est-à-dire l'équation (28) est accessible à la mesure expérimentale. La décomposition de la courbe de décroissance radioactive en la somme de quatre fonctions exponentielles donne les valeurs de s_1, s_2, s_3, s_4 et celles de $\alpha\lambda_1$, $\beta\lambda_2$, $\gamma\lambda_3$, $\delta\lambda_4$. Ces différents paramètres sont des fonctions plus ou moins complexes des vitesses v_1, v_2, v_3, v_T et des masses M, M_1, M_2, M_3.

Comme M est connu par le dosage de la calcémie et la mesure du volume sanguin, le système comporte sept inconnues.

L'équation du quatrième degré est facile à établir, les facteurs des diverses puissances de s ainsi que le terme constant de l'équation sont des fonctions simples des vitesses et des masses. Si on considère que l'équation du quatrième degré peut s'écrire $(s-s_1)\ (s-s_2)\ (s-s_3)\ (s-s_4) = 0$, en identifiant membre à membre les facteurs des diverses puissances de s et le terme constant de cette équation avec ceux de l'équation théorique, on obtient quatre relations entre les vitesses et les masses.

D'autre part, il ressort des équations (21), (24) et (25) que, compte tenu de ce que $\mu = 1$,

$$\lambda = \frac{\dfrac{v_1}{M_1} + s}{\dfrac{v_1}{M}} \tag{32}$$

Comme on connaît $\alpha\lambda_1$, $\beta\lambda_2$, $\gamma\lambda_3$, $\delta\lambda_4$, et s_1, s_2, s_3 et s_4 on peut calculer α, β, γ et δ. On écrit alors qu'au temps zéro: $R_1 = 0$, c'est-à-dire que $\alpha + \beta + \gamma + \delta = 0$ et on obtient une équation du troisième degré en v_1/M_1. Cette équation est la même en

v_2/M_2 si on raisonne sur π et en v_3/M_3 si on raisonne sur ρ. Les trois racines de cette équation représentent donc les valeurs de v_1/M_1, v_2/M_2, et v_3/M_3.

En reportant ces valeurs dans le système d'équations donné par l'identification des équations du quatrième degré, on obtient un système de quatre équations linéaires à quatre inconnues, qui est aisément soluble.

Interprétation de la décroissance monoexponentielle du segment DE

Le redressement de la courbe de décroissance de la radioactivité spécifique du calcium sérique vers le 8ème jour après l'injection (partie CD de la courbe de la Fig. 1) puis l'établissement d'une nouvelle décroissance monoexponentielle (segment DE) signifie qu'à ce moment un nouveau phénomène d'échange se manifeste. L'apparition tardive de ce phénomène ne peut s'expliquer que s'il est beaucoup plus lent que tous les autres.

En toute rigueur, on devrait donc ajouter aux quatre compartiments représentés sur la Fig. 3 un cinquième compartiment M_4 pour tenir compte de ce phénomène et raisonner sur le schéma représenté sur ce nouveau modèle.

La résolution pratique d'un tel système devient assez complexe; d'autre part il ne saurait être qu'exceptionellement question de poursuivre en clinique l'exploration pendant 18 jours, temps nécessaire à l'obtention d'une courbe complète. On peut toutefois mesurer commodément et avec une assez bonne précision la valeur de M_4 et la vitesse d'échange correspondante v_4 quand la courbe entière est connue, ou seulement le segment BCDE.

Les compartiments M, M_1, M_2 et M_3 s'équilibrent beaucoup plus vite entre eux que le compartiment M_4, de sorte que pour le raisonnement on peut admettre que l'échange avec M_4 porte sur l'ensemble du calcium du fonds commun P ($P = M + M_1 + M_2 + M_3$) et raisonner sur le modèle représenté sur la Fig. 4.

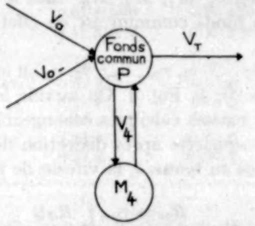

Fig. 4. Fonds commun calcique et échange lent: schéma simplifié.

Sur la Fig. 4, v_a et v_T ont la même signification que précédemment, v_4 représente la vitesse de l'échange le plus lent, M_4 la masse de calcium mise en jeu et P la masse du fonds commun.

Soit R la radioactivité totale du calcium du fonds commun au temps t et R_4 celle du calcium compris dans M_4. L'évolution du système pendant le temps dt est représentée par les deux équations différentielles:

$$\frac{\mathrm{d}R}{\mathrm{d}t} = -\frac{v_T + v_4}{P} R + \frac{v_4}{M_4} R_4 \tag{33}$$

$$\frac{\mathrm{d}R_4}{dt} = \frac{v_4}{P} R - \frac{v_4}{M_4} R_4 \tag{34}$$

La résolution de ce système conduit à des équations du type:

$$R = Ae^{-at} + Be^{-bt} \tag{35}$$

$$R_4 = A'e^{-at} - A'e^{-bt} \tag{36}$$

Nous avions proposé précédemment une méthode de calcul de v_4 et de M_4 à partir de ces équations, fondée sur la détermination de la tangente à l'origine et de l'asymptote de la courbe exprimant la décroissance de la radioactivité spécifique du calcium sérique en fonction du temps[1]. Cette méthode assez précise chez l'homme, puisque les deux droites sont données par les segments linéaires BC et DE de la courbe de la Fig. 1, l'est moins chez les animaux de laboratoire où le segment BC n'est jamais linéaire. Il est donc préférable d'appliquer le même raisonnement que dans le paragraphe *décomposition du fonds commun en ses constituants*. Ce système qui ne comprend que deux équations différentielles linéaires est beaucoup plus facilement soluble.

VÉRIFICATION DE LA THÉORIE CHEZ LE RAT

L'application de la méthode au rat permet de vérifier par la mesure expérimentale de la radioactivité fixée sur le squelette la validité du calcul de la vitesse de fixation physiologique du calcium sur l'os v_{o+}.

On injecte par voie intraveineuse 20 μC de $^{45}CaCl_2$ (environ 30 μg) et on dose la radioactivité spécifique du calcium sérique aux temps suivants: 2, 4, 6, 24, 32, 48 h. Le rat est d'autre part soumis à un bilan et on mesure la radioactivité totale de l'urine et des selles.

On obtient ainsi la partie BCDE de la courbe représentée sur la Fig. 1. En appliquant la théorie à cette partie de la courbe on peut calculer toutes les grandeurs définies ci-dessus sauf v_1, v_2, v_3, M_1, M_2, M_3. Chez le rat, l'homogénéisation de la radioactivité spécifique du fonds commun est en effet beaucoup trop rapide pour être facilement mesurable.

Pour calculer P, M_4, v_T, v_u, v_f, v_{o+}, v_{o-}, v_a, v_4 on applique la théorie correspondant au schéma représenté sur la Fig. 4. On sacrifie le rat après 72 h, c'est-à-dire quand la radioactivité des masses calciques échangeables devient très faible, et on mesure la radioactivité du squelette après dissection de l'animal. Si on appelle R_{os} la radioactivite fixée sur l'os au temps t, la vitesse de fixation sur l'os v_{o+} est reliée à R_{os} par la relation:

$$R_{os} = v_{o+} \int_0^t R_S \mathrm{d}t \tag{37}$$

La connaissance expérimentale de R_{os} et de la valeur de l'intégrale permet de calculer v_{o+}.

Nous rapportons dans le Tableau I la valeur de v_{o+} calculée des deux manières.

La concordance entre la vitesse de fixation du calcium sur l'os calculée par la théorie et celle mesurée expérimentalement justifie la plupart des hypothèses émises pour établir la théorie. En particulier la différenciation entre les processus irréversibles et réversibles est valable et tous les échanges sont *in vivo* entièrement réversibles.

APPLICATION DE LA MÉTHODE À L'HOMME

Nous rapportons dans le Tableau II trois exemples typiques concernant un sujet

TABLEAU I

COMPARAISON DE LA VALEUR DE LA VITESSE DE FIXATION PHYSIOLOGIQUE DU CALCIUM SUR L'OS, CALCULÉE D'APRÈS LA THÉORIE ET MESURÉE EXPÉRIMENTALEMENT

		v_{o+} *(calculée)* *(mg/jour)*	v_{o+} *(mesurée)* *(mg/jour)*
Rats rachitiques	No. 1	12.3	12.0
(4 mois, 70 g)	No. 2	14.3	15.2
	No. 3	17.4	17.4
Rats normaux	No. 1	53.0	52.8
(3 mois, 126 g)	No. 2	62.0	69.0

TABLEAU II

EXEMPLES D'APPLICATION DE LA MÉTHODE À L'HOMME

	Os normal	*Os hyperactif (maladie de Paget)*	*Os hypoactif (hypoparathyroïdie)*
Age	22 ans	57 ans	57 ans
Taille	1.82 m	1.75 m	1.65 m
Poids	70 kg	79 kg	61 kg
Sexe	Masculin	Masculin	Masculin
Volume sanguin	5.9 l	5.7 l	4.3 l
Calcémie	100 mg/l	105 mg/l	56 mg/l
v_1	134 g/jour	115 g/jour	36.4 g/jour
v_2	10.3 g/jour	78 g/jour	19.7 g/jour
v_3	2.22 g/jour	23 g/jour	1.7 g/jour
v_u (calciurie)	0.18 g/jour	0.34 g/jour	0.03 g/jour
v_f	0.12 g/jour	0.18 g/jour	0.24 g/jour
v_{o+}	0.88 g/jour	13.90 g/jour	0.53 g/jour
v_{o-}	0.87 g/jour	14.06 g/jour	0.54 g/jour
v_i (calcium ingéré)	0.92 g/jour	0.72 g/jour	0.79 g/jour
v_a (calcium absorbé)	0.31 g/jour	0.36 g/jour	0.26 g/jour
Taux d'absorption du calcium	34 %	50 %	33 %
M (calcium sanguin total)	0.32 g	0.34 g	0.13 g
M_1	1.52 g	0.88 g	0.31 g
M_2	1.30 g	6.46 g	1.01 g
M_3	3.43 g	22.83 g	1.70 g
P (fonds commun)	6.57 g	30.51 g	3.15 g

normal, un cas d'hyperactivité osseuse (maladie de Paget) et un cas d'hypoactivité osseuse (hypoparathyroïdie).

On injecte par voie intraveineuse 4 à 6 μC de $^{45}CaCl_2$ (environ 10 μg) et on dose la radioactivité spécifique du calcium sérique aux temps suivants: 5, 10, 20, 45 min, 1 h 15, 2, 3, 6, 24 h; par la suite on peut répéter les prélèvements de sang toutes les 24 h, ou mesurer la radioactivité spécifique du calcium urinaire. Le sujet est d'autre part soumis à un bilan et on mesure la radioactivité de l'urine et des selles.

Dans les cas où chez l'homme nous avons mesuré v_4, nous avons trouvé que le rapport v_4/v_{o+} est de l'ordre de 0.25 et le rapport M_4/P de l'ordre de 0.2.

Ces résultats permettent, sans nous attarder sur la signification des valeurs normales et sur leurs variations dans les cas pathologiques, de faire les remarques suivantes:

1. La théorie définit dans chaque cas l'existence de quatre compartiments quand on poursuit les mesures pendant 6 à 7 jours, de cinq si on les poursuit jusqu'à 18 jours. La première question qui se pose est de savoir si chacun des compartiments désigne la même entité chez tous les individus.

Si on se rapporte aux résultats du Tableau II on voit que dans les différents cas les masses d'un même compartiment et les vitesses d'échange correspondantes varient et quelquefois beaucoup. Un phénomène d'échange est caractérisé davantage par le rapport vitesse sur masse, c'est-à-dire la constante de vitesse, que par les valeurs absolues des vitesses et des masses. Nous avons donc représenté dans le Tableau III les valeurs des différentes vitesses d'échange et masses échangeables et celles du rapport vitesse sur masse.

TABLEAU III

COMPARAISON DES VITESSES D'ÉCHANGE ET DES MASSES DES COMPARTIMENTS DANS LES DIFFÉRENTS CAS

	v_1 *(g/jour)*	M_1 *(g)*	v_1/M_1	v_2 *(g/jour)*	M_2 *(g)*	v_2/M_2	v_3 *(g/jour)*	M_3 *(g)*	v_3/M_3
Os normal	134	1.52	88	10.3	1.30	7.9	2.22	3.43	0.65
Os hyperactif	115	0.88	130	78	6.46	12.1	23	22.83	1.01
Os hypoactif	36.4	0.31	117	19.7	1.01	19.5	1.7	1.7	1.00

Les différents rapports vitesse sur masse correspondant à un compartiment donné sont en meilleur accord que les valeurs absolues des deux grandeurs. D'autre part les processus d'échanges sont nettement distincts puisque les différents rapports v/M sont entre eux dans un rapport de 10 ou de 100.

On peut donc penser que les différents compartiments définis par l'analyse mathématique correspondent à des entités homogènes au point de vue de l'échange et que les variations observées dans les vitesses traduisent uniquement la différence des masses sur lesquelles les mêmes processus portent. Chaque compartiment désigne dans tous les cas la même entité.

2. Comme chez chaque individu le même compartiment désigne le même type de calcium échangeable, la deuxième question qui se pose est de savoir à quel système physiologique correspond chaque compartiment.

Le dosage du calcium dans les liquides interstitiels et les tissus, et les résultats obtenus dans les cas normaux et pathologiques permettent de penser que: (a) Le compartiment M_1, dont la vitesse d'échange avec le calcium sérique est la plus rapide, représente le calcium des liquides extracellulaires. (b) Le compartiment M_2 représente la fraction de calcium tissulaire la plus labile. (c) Le compartiment M_3 représente la fraction de calcium tissulaire la moins labile et la fraction de calcium osseux soumise à l'échange ionique. Nous n'avons pas, jusqu'à présent, pu différencier ces deux fractions. (d) Le compartiment M_4 dont la vitesse d'échange est très lente par rapport aux autres doit représenter la fraction de calcium osseux soumise au phénomène de recristallisation.

La définition du fonds commun, c'est-à-dire l'ensemble du calcium de l'organisme dont la radioactivité spécifique s'équilibre rapidement avec celle du sérum (moins de 48 h chez l'homme normal) exclut le compartiment M_4 qui ne peut être déterminé qu'en poursuivant les mesures pendant près de trois semaines.

3. Ces résultats justifient l'hypothèse selon laquelle v_{o-} ne porte pas, durant le temps de l'expérience, sur du calcium radioactif.

En effet si v_{o-} portait sur du calcium radioactif, la valeur calculée de v_{o+} correspondrait en réalité à $v_{o+} - v_{o-}$, c'est-à-dire que pour les cas rapportés dans le Tableau II les sujets seraient en bilan largement positif or ils sont en bilan équilibré.

On pourrait objecter enfin que v_{o-} ne porte qu'en partie sur du calcium radioactif, mais dans cette hypothèse, pour que la décroissance demeure monoexponentielle, il faudrait que le calcium détaché de l'os ait à chaque instant la même radioactivité spécifique que celui qui se fixe ou que celui du sérum, c'est-à-dire qu'une fraction constante du calcium fixé par formation d'os nouveau se détache immédiatement, ce qui physiologiquement paraît bien improbable.

4. Remarquons enfin que lorsqu'on ne calcule pas v_4 et M_4, ce qu'on appelle v_{o+} représente la somme de la vitesse de fixation physiologique du calcium sur l'os et de la vitesse de recristallisation. D'après les mesures que nous avons faites chez l'homme on peut admettre que la vitesse réelle de fixation physiologique du calcium sur l'os est égale à 0.75 v_{o+}. Cette remarque vaut également pour v_{o-}.

DISCUSSION

Précision de la méthode

L'équation de décroissance de la radioactivité spécifique du calcium sérique est obtenue par décomposition en quatre droites successives de la courbe expérimentale représentée en coordonnées semi-logarithmiques.

La précision correspondant à la première droite (segment BC de la Fig. 1 — terme $A_4 e^{-a_4 t}$ de l'équation (19)) est celle des dosages expérimentaux.

Les autres droites obtenues par différence comportent naturellement moins de précision. La deuxième et la troisième droite sont construites habituellement avec quatre points et la précision est toujours supérieure à 10 %.

En revanche la quatrième droite obtenue le plus souvent avec deux points (0 et 5 min) comporte une part appréciable d'indétermination. Tout d'abord l'ordonnée au temps zéro est obtenue en divisant la radioactivité injectée par le calcium sanguin total calculé d'après la calcémie et le volume sanguin. Il est bien évident que ceci ne correspond pas à la réalité puisque l'homogénéisation dans le sang du calcium injecté exige un certain temps.

Nous avons déterminé chez l'homme l'allure de la courbe d'homogénéisation, la Fig. 5 exprime les résultats.

On voit sur cette figure que la courbe passe, environ 45 sec après l'injection, par un maximum qui atteint 80 % de la valeur théorique maximale. On devrait donc prendre le temps 45 sec comme temps initial et 80 % de la radioactivité spécifique théorique maximale comme radioactivité spécifique initiale. Mais si on remarque que la décroissance de la radioactivité spécifique entre 50 et 80 sec est pratiquement linéaire, on voit qu'en extrapolant cette droite à l'origine, elle coupe l'axe des ordonnées en un point très voisin de 100 %. L'erreur commise dans la détermination de la radioactivité spécifique initiale, telle qu'elle a été définie ci-dessus, est donc très faible.

Toutefois l'équation de la quatrième droite, qui est obtenue par deux valeurs résultant de trois différences, ne saurait être très précise. Il est donc important de savoir quelle répercution ont les valeurs de A_1 et de a_1 du terme $A_1 e^{-a_1 t}$ de l'équation

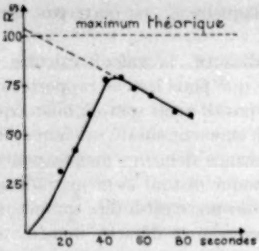

Fig. 5. Représentation de la variation de la radioactivité spécifique du calcium sérique de 20 à 80 sec après l'injection.

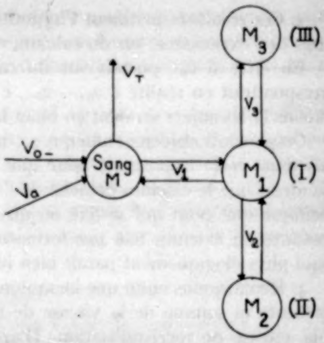

Fig. 6. Variante du modèle de décomposition du fonds commun calcique en ses constituants.

(19) sur l'ensemble des résultats. Les variations de ces valeurs portent surtout sur v_1 et à un moindre degré sur M_1. Ainsi pour une variation de pente de 35 %, ce qui dépasse le maximum d'erreur possible, la variation de v_1 est de l'ordre de 50 %, celle de M_1 de 30 %, celle des autres grandeurs est inférieure à 8 %. Si l'on veut connaître avec plus de précision v_1 et M_1, il faut donc multiplier le nombre des points intermédiaires dans les temps inférieurs à 5 min pour obtenir la quatrième droite avec une meilleure précision.

En conclusion on peut dire que la méthode décrite ici donne v_1 et M_1 à moins de 20 % près et les autres valeurs à moins de 10 %.

Relations entre les compartiments

L'analyse mathématique de la courbe de décroissance de la radioactivité spécifique du calcium sérique ne donne aucun renseignement sur la disposition relative des différents compartiments. Théoriquement un grand nombre de modèles est concevable, parmi lesquels il faut retenir ceux qui sont compatibles avec les données physiologiques.

La première question à résoudre est de savoir à partir de quel(s) compartiment(s) s'effectuent les sorties irréversibles du calcium. Il est évident que seul le calcium sérique ou extracellulaire entre en considération. L'intensité de l'irrigation sanguine du rein, de l'intestin et de l'os conduit à penser que la sortie de calcium s'effectue de façon prépondérante à partir du sang.

En ce qui concerne la disposition des compartiments, le schéma le plus logique est celui qui est représenté sur la Fig. 2, où tous les compartiments sont en équilibre avec le sang. On ne peut toutefois exclure une autre disposition, où le calcium des liquides extracellulaires occuperait une position centrale et serait en échange direct avec le calcium du sang, des tissus et de l'os. Ce modèle est représenté sur la Fig. 6.

On peut calculer les vitesses et les masses selon ce modèle en employant une théorie semblable à celle décrite précédemment. Nous rapportons sur le Tableau IV les résultats concernant le cas normal rapporté sur le Tableau II, calculés selon les deux modèles.

Les valeurs calculées selon l'un ou l'autre modèle sont très voisines; en l'absence

TABLEAU IV

COMPARAISON DE LA GRANDEUR DES COMPARTIMENTS CALCIQUES
ET DES VITESSES D'ÉCHANGES SELON LE MODÈLE CHOISI

	Modèle Fig. 2	*Modèle Fig. 6*
v_1	134 g/jour	149 g/jour
v_2	10.3 g/jour	9.6 g/jour
v_3	2.2 g/jour	2.2 g/jour
v_T	1.18 g/jour	1.22 g/jour
M	0.32 g	0.32 g
M_1	1.52 g	1.83 g
M_2	1.30 g	1.19 g
M_3	3.43 g	3.29 g
P	6.57 g	6.63 g

d'arguments plaidant en faveur du modèle représenté sur la Fig. 6, nous avons adopté le modèle représenté sur la Fig. 2.

Méthode simplifiée

Un certain nombre d'auteurs[2–9] et nous-mêmes avons proposé de mesurer le fonds commun calcique et la vitesse de fixation du calcium sur l'os en raisonnant simplement sur la partie BC de la courbe représentée sur la Fig. 1. Cette méthode simplifiée, qui pourrait être d'un emploi assez commode en clinique, donne toutefois des résultats entachés d'erreurs dont il faut tenir compte.

Prenons comme exemple le cas normal, dont les constantes calculées selon la méthode complète sont rapportées dans le Tableau II. L'équation de décroissance de la radioactivité spécifique du calcium sérique, en supposant une radioactivité initiale de 1,000, est entre 0 et 7 jours (*t* exprimé en heures):

$$R_S = 850\ \mathrm{e}^{-23t} + 62.8\ \mathrm{e}^{-0.55t} + 52.6\ \mathrm{e}^{-0.062t} + 34.6\ \mathrm{e}^{-0.0064t} \tag{38}$$

Lorsqu'on raisonne uniquement sur la partie linéaire de la courbe de décroissance, on néglige dans l'équation (38) les trois premiers termes et on admet que l'équation de décroissance est:

$$R_S = 34.6\ \mathrm{e}^{-0.0064t} \tag{39}$$

Si on calcule le fonds commun à partir de cette équation, la calcémie totale étant de 0.32 g, on trouve:

$$P = \frac{1,000 \times 0.32}{34.6} = 9.2\ \mathrm{g}$$

et la vitesse totale de sortie v_T du calcium hors du fonds commun est:

$$9.2 \times 0.0064 \times 24 = 1.41\ \mathrm{g/jour}.$$

Or les chiffres obtenus par la méthode complète sont 6.57 g pour le fonds commun et 1.18 g/jour pour la vitesse de sortie du calcium.

L'erreur commise est donc:

$$\frac{1.41 - 1.18}{1.18} = 0.195 \text{ soit } 19.5\,\% \text{ pour } v_T$$

$$\frac{9.2 - 6.57}{6.57} = 0.40 \text{ soit } 40\,\% \text{ pour } P.$$

Ces erreurs ne sont pas négligeables. D'autre part, elles varient avec l'équation de décroissance, ce qui complique l'interprétation des résultats relatifs aux cas normaux et pathologiques.

On peut expliquer l'erreur commise en se fondant sur les considérations suivantes. L'équation réelle de décroissance de la radioactivité spécifique est:

$$R_S = A_1 e^{-a1t} + A_2 e^{-a2t} + A_3 e^{-a3t} + A_4 e^{-a4t} \tag{19}$$

Lorsqu'on emploie la méthode simplifiée, on admet que la loi de décroissance est:

$$R_S = A_4 e^{-a4t} \tag{40}$$

Représentons graphiquement sur la Fig. 7 les fonctions (19) et (40). La courbe ACD correspond à l'équation (19), la courbe BCD à l'équation (40), les deux courbes sont pratiquement confondues à un temps t_1, qui expérimentalement est de l'ordre de 48 h.

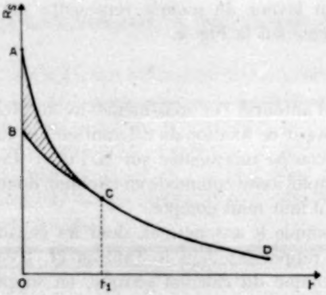

Fig. 7. Représentation des fonctions complète et simplifiée exprimant la variation de la radioactivité spécifique du calcium sérique en fonction du temps.

Si on appelle Σ la surface délimitée par la courbe ACD et les axes de coordonnées; la vitesse de sortie du calcium hors du fonds commun v_T est égale, d'après l'équation (6), à:

$$v_T = \frac{R_t}{\Sigma} \tag{41}$$

Appelons σ la surface délimitée par la courbe BCD et les axes de coordonnées; lorsqu'on emploie la méthode simplifiée, la vitesse v'_T que l'on mesure est égale à:

$$v'_T = \frac{R_t}{\sigma} \tag{42}$$

et l'erreur relative commise Δ est:

$$\Delta = \frac{v'_T - v_T}{v_T} = \frac{\frac{R_t}{\sigma} - \frac{R_t}{\Sigma}}{\frac{R_t}{\Sigma}} = \frac{\Sigma - \sigma}{\sigma} \tag{43}$$

c'est-à-dire que l'erreur relative est représentée par le rapport entre la surface du triangle courbe ABC et celle délimitée par la courbe BCD et les axes de coordonnées.

Si on se reporte à l'équation (19), la surface correspond à l'intégrale de la fonction, c'est-à-dire:

$$\Sigma = \left[-\frac{A_1}{a_1} e^{-a1t} - \frac{A_2}{a_2} e^{-a2t} - \frac{A_3}{a_3} e^{-a3t} - \frac{A_4}{a_4} e^{-a4t} \right]_0^\infty \tag{44}$$

soit:

$$\Sigma = \frac{A_1}{a_1} + \frac{A_2}{a_2} + \frac{A_3}{a_3} + \frac{A_4}{a_4} \tag{45}$$

De même d'après l'équation (40):

$$\sigma = \frac{A_4}{a_4} \tag{46}$$

et l'erreur relative est égale à:

$$\Delta = \frac{\dfrac{A_1}{a_1} + \dfrac{A_2}{a_2} + \dfrac{A_3}{a_3}}{\dfrac{A_4}{a_4}} \tag{47}$$

Dans l'exemple que nous avons choisi:

$$\frac{A_1}{a_1} = 37, \frac{A_2}{a_2} = 116, \frac{A_3}{a_3} = 847, \frac{A_4}{a_4} = 5{,}410$$

$$\Delta = \frac{37 + 116 + 847}{5{,}410} = \frac{1{,}000}{5{,}410} = 0.185$$

L'erreur commise dans ce cas sur la vitesse v_T est donc de 18.5 %.

La plupart du temps les auteurs se contentent d'extrapoler à l'origine le segment BC de la courbe représentée sur la Fig. 1, ils déterminent ainsi la radioactivité spécifique initiale, qui, étant donné la radioactivité injectée, leur permet de calculer le fonds commun. Il est évident que cette méthode comprend déjà l'erreur signalée ci-dessus. En toute logique, si on voulait garder le principe de ce calcul, on devrait soustraire de la radioactivité injectée la radioactivité qui sort du fonds commun pendant les 48 premières heures et diviser la radioactivité restante par la radioactivité spécifique du calcium à ce moment. Mais même dans ce cas le chiffre obtenu ne correspondrait pas exactement à la réalité; les trois compartiments définis plus haut ne s'équilibrent pas à la même vitesse avec le sang et la radioactivité spécifique du calcium de chacun de ces compartiments n'est jamais exactement celle du calcium sérique.

Reprenons notre exemple, on peut calculer, une fois connues les masses et les vitesses, la décroissance de la radioactivité spécifique du calcium de chacun des trois compartiments d'après les équations (29), (30) et (31). Si on appelle R_{s_M} la radioactivité spécifique du calcium sérique, $R_{s_{M_1}}$ celle du compartiment M_1, $R_{s_{M_2}}$ celle du compartiment M_2 et $R_{s_{M_3}}$ celle du compartiment M_3, les lois de décroissance des radioactivités spécifiques sont:

$$R_{s_M} = 850\, e^{-23t} + 62.8\, e^{-0.55t} + 52.6\, e^{-0.062t} + 34.6\, e^{-0.0064t} \tag{38}$$

$$R_{s_{M_1}} = -162\, e^{-23t} + 74\, e^{-0.55t} + 53.3\, e^{-0.062t} + 34.7\, e^{-0.0064t} \tag{48}$$

$$R_{s_{M_2}} = -11.7\, e^{-23t} - 87.3\, e^{-0.55t} + 63.3\, e^{-0.062t} + 35.7\, e^{-0.0064t} \tag{49}$$

$$R_{s_{M_3}} = -1.0\, e^{-23t} - 2.8\, e^{-0.55t} - 41.4\, e^{-0.062t} + 45.2\, e^{-0.0064t} \tag{50}$$

Les Fig. 8 et 9 représentent la variation de la radioactivité spécifique moyenne du calcium des différents compartiments, exprimée par les équations (38), (48), (49) et (50).

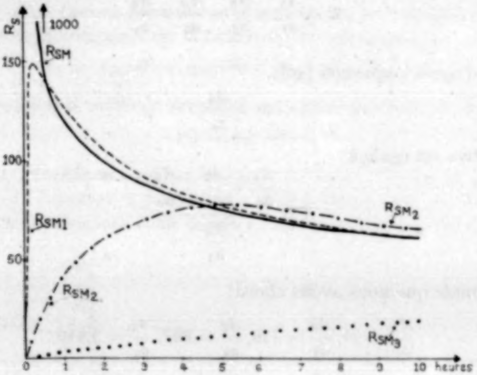

Fig. 8.

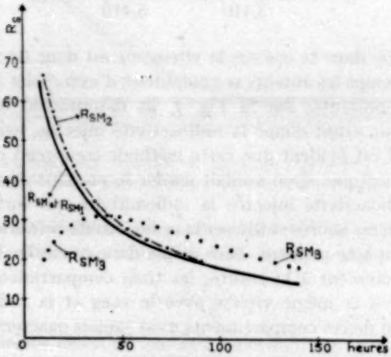

Fig. 9.

Figs. 8 et 9. Représentation de la variation en fonction du temps des radioactivités spécifiques du calcium des différents compartiments (cas normal).

Pour les trois compartiments, l'évolution de la radioactivité spécifique du calcium est du même type: elle augmente, passe par un maximum, atteint celle du sang, la dépasse puis tend à nouveau à s'équilibrer avec elle.

La radioactivité spécifique du calcium extracellulaire demeure pratiquement identique à celle du sang à partir de 10 h après l'injection. Celle du calcium tissulaire atteint celle du sang à la 5ème h puis lui demeure supérieure jusqu'à la 100ème h. La radioactivité spécifique du calcium du compartiment M_3 s'équilibre beaucoup plus lentement avec celle du sérum; elle la dépasse après 40 h et reste supérieure par la suite.

Quand on considère donc le fonds commun comme un ensemble homogène ayant une radioactivité spécifique définie, on commet toujours, même dans les temps longs, une légère erreur surtout en ce qui concerne le compartiment M_3. Cette erreur, d'autre part, varie naturellement avec l'équation de la courbe de décroissance.

Il est évident que plus le fonds commun est grand, plus l'erreur est grande. Ainsi pour un fonds commun de 3.5 g l'erreur est de 35.8 % et de 18 % pour la vitesse; pour un fonds commun de 6.6 g l'erreur est de 40 % et de 19.5 % pour la vitesse; enfin dans les fonds communs très grands, comme dans le cas d'hyperactivité osseuse rapporté dans le Tableau I, l'erreur commise serait de 96.5 % pour le fonds commun et de 38.6 % pour la vitesse.

Cela ne veut pas dire qu'il faille rejeter systématiquement l'emploi de la méthode simplifiée, qui dans beaucoup de cas doit pouvoir rendre les plus grands services en clinique. Il faut simplement, lorsqu'on emploie cette méthode, affecter les valeurs calculées d'un facteur de correction.

RÉSUMÉ

Nous proposons une méthode de mesure des principales voies du métabolisme calcique chez l'homme fondée sur les techniques et la théorie suivantes: (1) Injection intraveineuse d'une quantité pondéralement négligeable de $^{45}CaCl_2$. (2) Déterminations de la radioactivité spécifique du calcium sérique, de la radioactivité totale des urines et des selles et dosage du calcium ingéré et excrété. (3) Analyse mathématique de la courbe de décroissance de la radioactivité spécifique du calcium sérique.

Nous pouvons ainsi connaître: (a) Le taux de l'absorption intestinale du calcium dans l'état chimique où il se trouve dans les aliments. (b) La quantité de calcium excrétée par l'intestin en la différenciant de celle non absorbée. (c) Les vitesses des processus physiologiques de renouvellement du squelette par ostéolyse et ostéoblastose. (d) La masse de calcium rapidement échangeable de l'organisme, c'est-à-dire le fonds commun calcique. Ce fonds commun est décomposable en trois masses de calcium dont, les constantes de vitesse d'échange avec le calcium sérique sont très différentes et qui sont identifiables à des entités anatomo-physiologiques définies. (e) Les vitesses d'échange avec le calcium sérique du calcium de ces différents constituants du fonds commun. (f) La masse de calcium osseux soumis au phénomène de recristallisation.

Nous avons rapporté des exemples d'application de la méthode à l'homme et la vérification de la théorie chez le rat. Nous avons discuté la précision de la méthode et celle de la méthode simplifiée habituellement employée.

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A NEW COLORIMETRIC METHOD FOR THE DETERMINATION OF KETOHEXOSES IN PRESENCE OF ALDOSES, KETOHEPTOSES AND KETOPENTOSES

ZACHARIAS DISCHE AND ANIMA DEVI

Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y. (U.S.A.)

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SUMMARY

1. A modification of the cysteine–H_2SO_4 reaction of carbohydrates is described which permits quantitative determinations of 10 μg/ml of ketohexose in large excess of other ketoses and aldoses.

2. This procedure permits to differentiate between sorbose and other ketohexoses.

INTRODUCTION

Methods for the determination of ketohexose in the range of concentration between 1 and 100 μg/ml so far described in the literature are general reactions of keto sugars in which ketoheptoses and ketopentoses often show higher extinction coefficients than ketohexoses. The determination of the latter, therefore, in solutions in which other ketoses are present often presents a difficult analytical problem. This becomes particularly disturbing when the formation of ketohexoses form either ketopentoses or ketoheptoses has to be studied. The present report deals with a modification of the cysteine-sulfuric acid reaction of carbohydrates[1] which permits quantitatively to determine ketohexoses in presence of even large excess of ketopentose and ketoheptose.

METHODS

Preparations

Preparations of fructose, sorbose, fructose-6-phosphate and fructose-1,6-diphosphate were commercial preparations. The amounts of fructose in the two esters were estimated by comparing the optical density in the cysteine-carbazole reaction at 560 mμ with that of fructose[1]. The preparation of tagatose was obtained from the laboratory of DR. LARDY*, the preparation of sedoheptulose-7-phosphate from the laboratory of DR. HORECKER*. The content in sedoheptulose in this preparation was determined by the cysteine-H_2SO_4 (see ref. [2]) reaction of heptoses using as

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* We are greatly indebted to Dr. H. LARDY for the preparation of tagatose and to Dr. B. L. HORECKER for a sample of sedoheptulose-7-phosphate.

standard a solution of crystalline sedoheptulosan. Glucoheptulose, glucose, galactose and mannose were commercial preparations and were not standardized. Ribose-5-phosphate (Schwartz Broths. Laboratories) was isomerized by incubation in a blood hemolysate to the equilibrium mixture of this ester with the two ketopentose-5-phosphates[3] and the TCA filtrate of the hemolysate was used as preparation of the equilibrium mixture.

Procedure

To 0.5 ml of a solution containing 10–100 μg of ketohexose is added 0.1 ml of a freshly prepared 25 % solution of cysteine-hydrochloride and 6 ml of a 75 % by volume of H_2SO_4 (prepared by adding 450 ml of concentrated sulfuric acid to 190 ml of water). The sample is shaken and left at room temperature. A yellow color appears after a few min, increases in intensity for about 3 h, and remains practically constant for about 2 h. The color shows an absorption maximum between 410 and 414 mμ. A blank containing water instead of the sugar solution is prepared in the same way.

Specificity of the reaction

Aldohexoses react in an identical way, but very much weaker. Solutions of glucose, galactose and mannose containing 1 mg/ml of sugar do not show any discernible yellow color after 3 h. Ketopentoses and ketoheptoses show under the conditions of this reaction a red color which develops very slowly and at concentrations of about 200 μg/ml is clearly discernible only after 24 h. Lower sugars and hexuronic acids do not react at all. The yellow color of ketohexose changes slowly to a blue color, which is particularly conspicuous in the case of sorbose. DNA gives the well-known characteristic pink color with an absorption maximum of 490 mμ which reaches its maximum already after about 10 min. At the concentration of 0.1 %, glucose but not galactose or mannose shows a very weak blue color after 24 h.

Absorption spectra

The complete absorption spectra are presented in Fig. 1. As can be seen, all keto sugars give absorption curves with two maxima, one in the visible light and one in the u.v. The absorption maxima for ketopentoses and ketoheptoses in the visible light at 510 and 540 mμ respectively are so low at concentrations used for the preparations of the absorption curves after 4 h that they were not registered in the curves. Ketohexoses have one absorption maximum between 410 and 414 mμ, a second absorption maximum at 298 mμ, and in addition, a third maximum at 605 mμ. This last maximum apparently corresponds to the maxima of ketopentoses and ketoheptoses in the visible light. Sorbose differs significantly from fructose and tagatose by a low additional maximum at 360 mμ, whereas the two other ketohexoses show a minimum at 335 mμ. The absorption in the u.v. develops much slower than the absorption at 414 mμ and reaches its maximum after about 20 h. During the period between 4 and 20 h, the absorption at 414 mμ decreases continuously. The absorption in the u.v. of glucoheptulose and sedoheptulose increases slowly during the 20 h period with an absorption maximum between 307 and 310 mμ. The absorption of ketopentose was followed only down to 265 mμ, at which wave length the maximum was not yet reached.

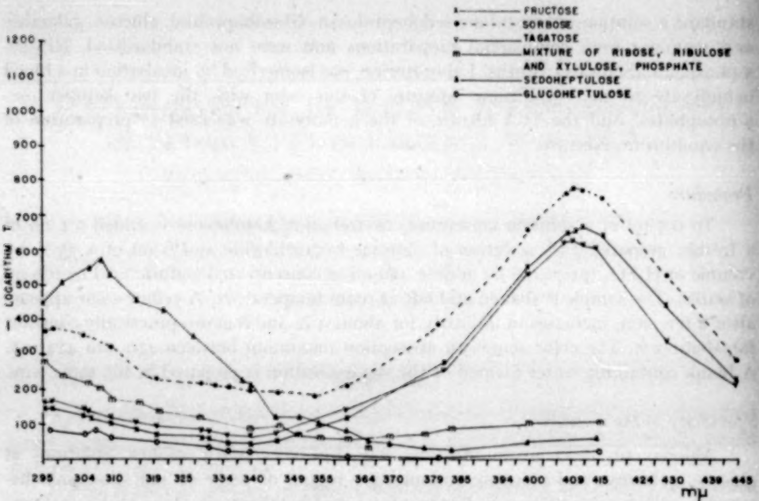

Fig. 1. Absorption spectra of various ketoses in the cysteine–H_2SO_4 reaction.

Determination of fructose and its phosphate esters in presence of ketopentose and ketoheptose esters

As can be seen from Table I, the optical density of solutions of fructose as well as fructose-6-phosphate in the cysteine–H_2SO_4 reaction is proportional to the concentration of fructose-6-phosphate in the range between 2.5 and 10 mg/100 ml. At 412 mμ, however, sedoheptulose phosphate as well as the ketopentose phosphates show a significant optical density which in presence of an excess of these sugars might interfere with the determination of ketohexose. This interference can, however, be eliminated by using the differences of O.D. at 412 and 380 mμ. These differences are for ketohexoses only 40 % lower than O.D.$_{412}$ itself but becomes negligible for ketoheptose and negative for ketopentose. This negative value amounts to no more than 1.5 % of the value for equivalent amounts of ketohexose. It must be noted, however, that the wave lengths at which the O.D. of ketoheptose solutions equals that at 412 mμ, can vary by a few mμ from one experiment to another. In carrying out, therefore, determinations of ketohexose or its esters in presence of ketoheptose and ketopentose, a standard of ketoheptose present in the solution should be used along with the experimental samples, and in every case the wave length should be found at which the O.D. for ketoheptose equals that at 412 mμ. In addition, the values obtained should be corrected for ketopentose. This correction in general will be very small and it is not necessary to use a standard of ketopentose with each determination. It should be noted that sedoheptulosan cannot be used as standard for sedoheptulose as it reacts very weakly in the cysteine–H_2SO_4 reaction in comparison with sedoheptulose itself or its phosphate.

TABLE I

OPTICAL DENSITIES (O.D.) AT 380 AND 412 mμ AND THEIR DIFFERENCES IN THE CYSTEINE–H_2SO_4 REACTION OF VARIOUS KETO AND ALDO SUGARS

Expt. No.	*Substance*	*Concentration mg/100 ml of sugar*	*O.D.$_{380}$ × 1000*	*O.D.$_{412}$ × 1000*	*O.D.$_{412}$ — O.D.$_{380}$ × 1000*
I	a. Fructose	10	272	660	388
	b. Fructose	5	140	332	192
	c. Fructose	2.5	70	168	98
	d. Fructose-6-phosphate	10	305	687	382
	e. Fructose-6-phosphate	5	150	340	190
	f. Fructose-1-phosphate	4.4	110	270	160
	g. Fructose-1,6-diphosphate	15	404	1000	596
	h. Tagatose	10	274	630	356
	i. Sorbose	10	396	754	358
	j. Glucoheptulose	20	9	13	4
II	a. Fructose	10	279	638	359
	b. Glucose	100	14	42	28
	c. Galactose	100	10	28	18
	d. Mannose	100	8	32	24
III	a. Sedoheptulose-7-phosphate	10	47	45	—2
	b. Equilibrium mixture of ribose-5-phosphate and its 5-ketopentose phosphate isomers	100	93	84	—9

DISCUSSION

The reaction with cysteine and H_2SO_4 of ketohexose seems to be particularly useful in studies on the interconversion between hexoses and pentose in living cells, as in these reactions we are always confronted with mixtures of all three types of ketoses, and under experimental conditions the amounts of ketoheptose and ketopentose may by far exceed the amount of ketohexose. In the cysteine–H_2SO_4 reaction, the determination of ketohexoses is not interfered with even by much larger amounts of sedoheptulose phosphate or ketopentose-5-phosphate, as the O.D.'s due to the different types of ketoses were found to be either negligible or very small and additive.

The cysteine–H_2SO_4 reaction, furthermore, may become useful for the differentiation between different ketohexoses. Fructose, sorbose and tagatose, for instance, show nearly identical values for the difference of extinction coefficients at 412 and 380 mμ. When the reaction mixtures are left for 20 h at room temperature, the O.D.$_{606}$ due to the development of the blue component, is about 7 times as high for sorbose than for the two other ketohexoses. The development of this blue chromogen also strongly depends on the esterification of the fructose with phosphate. In fructose-6-phosphate, for instance, the extinction coefficient at 605 mμ is only half as high as that for fructose, whereas in fructose-1-phosphate on the contrary, a higher extinction coefficient is found at this wave length. Fructose-1,6-diphosphate consequently shows intermediate values between fructose-1- and fructose-6-phosphate. The difference in the development of the blue color between fructose-6-phosphate and fructose is noticable even with the naked eye. Sorbose also is characterized by a shift of the absorption maximum at 412 mμ to 390 mμ on standing, which does not take place in the case of fructose, its esters, and of tagatose.

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A COLORIMETRIC PROCEDURE FOR THE DETERMINATION OF TRIOSE PHOSPHATE AND FRUCTOSE-1,6-DIPHOSPHATE IN PRESENCE OF OTHER SUGARS

ZACHARIAS DISCHE AND EDITH LANDSBERG
Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y. (U.S.A.)

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SUMMARY

1. A colorimetric method is described which permits to determine 0.1 μmole/ml of fructose-1,6-diphosphate and triose phosphate.

2. The method is based on the ability of dihydroxyacetone phosphate to condense in the presence of muscle aldolase with formaldehyde to form erythrulose-1-phosphate.

3. In the presence of a large excess of formaldehyde and of hexose-6-phosphate isomerase, the conversion of fructose diphosphate and triose phosphate to erythrulose-1-phosphate is practically complete.

4. The erythrulose-1-phosphate is determined by the cysteine–H_2SO_4 reaction of tetroses using an incubation mixture of fructose diphosphate with formaldehyde and muscle aldolase as standard.

INTRODUCTION

The determination of triose phosphate and fructose-1,6-diphosphate is mostly carried out enzymically by determining the first compound by the reduction of DPN in the presence of triose phosphate dehydrogenase or the oxidation of $DPNH_2$ in presence of glycerol phosphate dehydrogenase. The fructose diphosphate is then determined after conversion to glyceraldehyde-3-phosphate by muscle aldolase and triose phosphate isomerase. This method requires enzyme preparations of high purity, as it is based on the determination of the increase or decrease of $DPNH_2$ which appears as intermediate in many other enzyme reactions. The present report deals with a rather simple procedure based on colorimetry of the end product of the reaction between formaldehyde and dihydroxyacetone phosphate catalyzed by muscle aldolase preparations containing triose phosphate isomerase. In this procedure the purity of the muscle aldolase preparation does not appear to be critical.

EXPERIMENTAL

Principle

Formaldehyde condenses in presence of muscle aldolase with dihydroxyacetone phosphate to erythrulose-1-phosphate[1]. This condensation proceeds to an equilibrium according to the equation (Formaldehyde)(Dihydroxyacetone P) = *K* (Erythrulose-1-P). When the concentrations of the reactants exceed a certain level and formaldehyde is present in large excess over the triose phosphate the equilibrium is shifted almost completely towards erythrulose-1-phosphate and only negligible amounts of triose phosphate and fructose diphosphate remain in solution. The decrease in formaldehyde under these conditions is also negligible. If the preparation of muscle aldolase contains a sufficient amount of triose phosphate isomerase, the glyceraldehyde-3-phosphate formed by muscle aldolase is converted to dihydroxyacetone phosphate at a rate corresponding to the disappearance of the latter ester. Triose phosphate is formed from FDP as soon as it is used up in the reaction. When the reaction is completed the amount of erythrulose-1-phosphate is proportional to the sum of FDP and triose phosphate present in solution.

Procedure for the determination of the sum of FDP and triose phosphate

To 1 ml of an experimental sample containing 0.1 to 2 μmole/ml of triose phosphate or its equivalent of FDP are added 2 ml of 0.01 *M* solution of phosphate buffer of pH 7.4, containing about 1.5 ml of a commercial muscle aldolase preparation (Nutritional Biochemical) per 100 ml of buffer. Afterwards 1 ml of the same buffer containing 0.1 % of formaldehyde are added, and the sample stirred, tightly stoppered, incubated and left for 6 h at room temperature. A standard solution containing 1 μmole/ml FDP and a blank containing the solvent of the experimental sample are run simultaneously in the same way. In addition, controls of one experimental sample, one standard and one blank are set up in exactly the same way, except that instead of the 2 ml of the phosphate buffer containing muscle aldolase, 2 ml of a pure phosphate buffer are added. When the determination is carried out on tissue extracts, the latter should be deproteinized with TCA and not with perchloric acid, which after neutralization even in very low concentration interferes with the activity of muscle aldolase. The TCA filtrates should be neutralized with solid $NaHCO_3$ or $KHCO_3$. At the end of the incubation period 0.15 ml of a 100 % TCA solution (100 g TCA in 100 ml of solution) are added under stirring, and the samples kept well stoppered over night at 4°. The samples containing aldolase are then centrifuged and the supernatant used for the analysis. The cysteine–sulfuric acid reaction of tetrose[2] is then carried out on all six samples. All samples are read in the Beckman spectrophotometer at 458 and 480 mμ against corresponding blanks. From the difference $E_{458} - E_{480}$ which is proportional to the amount of tetrose formed in the experimental sample and standard incubated with aldolase, are subtracted the values obtained from the corresponding control samples. The ratio of the value in the experimental sample divided by the value obtained with the standard multiplied by the concentration of the standard gives the concentration of the sum of FDP and triose phosphate present in the experimental sample.

Determination of FDP alone

If FDP is present in a solution containing muscle aldolase, it will be in equilibrium with triose phosphate. The amount of FDP alone can then be determined in the same way as the sum of FDP and triose phosphate by preincubating the experimental sample, the standard and the blank, as well as the corresponding controls, for 20 min with 0.5 N NaOH and neutralizing the solution afterwards with H_2SO_4 or TCA. During this incubation in alkaline solution, triose phosphate is completely destroyed, whereas only 5 % of the FDP in the experimental sample as well as in the standard solution are lost. The incubation with formaldehyde in presence of muscle aldolase of samples preincubated with NaOH, therefore, will yield the amount of erythrulose-1-phosphate corresponding to the amount of FDP originally present in solution.

RESULTS

Measurements of the optical density with the cysteine–sulfuric acid reaction of erythrulose-1-phosphate formed from FDP solution of concentrations varying between 0.1 and 2 µmole/ml show a satisfactory proportionality between the concentration of the sugar and the optical density in the cysteine–sulfuric acid reaction of the samples. The results of these determinations are listed in Table I. To determine how far it is of importance for this determination whether FDP is present as such in solution or in equilibrium with TrP, two determinations were carried out in such a way that FDP was incubated at 33° with muscle aldolase until the equilibrium between FDP and TrP was established, the sample deproteinized with TCA, and the sum of FDP and TrP determined. Simultaneously such a determination was carried out on the same sample not incubated with muscle aldolase. As can be seen from Table I, no significant difference appeared between these two samples. To determine how far correct values can be obtained with this procedure for the amounts of FDP present in equilibrium with triose phosphate in the presence of muscle aldolase, a solution containing 1.5 µmole/ml of FDP was incubated with a human hemolysate at 33° for 30 min until an equilibrium between FDP and triose phosphate catalyzed by the muscle aldolase present in the hemolysate has been established. The hemolysate

TABLE I

PROPORTIONALITY BETWEEN AMOUNTS OF FDP AND THOSE OF ERYTHRULOSE-1-PHOSPHATE FORMED FROM THIS ESTER IN PRESENCE OF FORMALDEHYDE AND MUSCLE ALDOLASE

Expt. No.	*FDP µmole/ml*	$(E_{418} - E_{490}) \times 1000$	
		Solution not preincubated with aldolase	*Solution preincubated with aldolase in hemolysates*
I	0.8	480	
	0.4	238	244
	0.2	120	
II	0.4	270	
	0.2	130	
	0.1	70	
III	0.8	450	448
	0.4	220	

was deproteinized with TCA and the amount of triose phosphate present in solution was determined as alkali-split P. It represented 57.5 % of the total phosphorus present in the FDP. The FDP, therefore, present in solution corresponded to 42.5 % of the originally present amount. The FDP in equilibrium was then determined in two such experiments by the formaldehyde aldolase procedure. It yielded 42 and 40.5 % of FDP in the two experiments, in satisfactory agreement with the direct determination by phosphate hydrolysis.

DISCUSSION

The procedure described here promises to be particularly useful in those cases in which the problem is the determination of the total amount of TrP formed in enzyme reactions. The results obtained with this procedure will be independent of side reactions due to the condensation of dihydroxyacetone phosphate with other aldehydes formed in metabolic processes, like tetroses or glycolic aldehyde. Generally the products of such condensations will also be broken up in the presence of large excess of formaldehyde to dihydroxyacetone phosphate and the corresponding aldehyde and TrP then will finally appear in form of tetrose-1-phosphate. On the other hand, formation of significant amounts of such side products could effect the determination of FDP which is in equilibrium with TrP in solution. Sedoheptulose-1,7-phosphate for instance, formed from E-4-P and dihydroxyacetone phosphate would not be destroyed by incubation with alkali at room temperature, but would in the subsequent determination of the non-dissociated FDP add its TrP to that yielded by FDP, thus increasing the apparent amount of the latter present in the solution. It will, therefore, often be necessary in such determinations either to exclude the presence of other condensation products of dihydroxyacetone phosphate with aldehydic compounds, or first determine these side products and account for their contributions to the results of the determinations.

The procedure described here for the determination of TrP appears to be highly specific insofar as it is based on two characteristics of dihydroxyacetone phosphate, namely (1) to condense with aldehydic compounds in presence of muscle aldolase, and (2) to yield with formaldehyde a compound which shows the characteristic color reaction of tetroses. It seems unlikely that any other compound but dihydroxyacetone phosphate would simultaneously behave in this way. This seems to represent a certain advantage against those methods of determination of TrP which rely on the determination of reduced DPN and do not characterize the end product of the enzymic reaction.

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Short Communications

The nature of bone citrate

Relatively large amounts of citrate are present in bone[1,2] but, in view of its large size, it is generally agreed that the citrate ion cannot occur within the apatite lattice of the bone crystals. From theoretical considerations Hendricks and Hill[3] and Hendricks[4] have concluded that citrate is adsorbed on the crystal surfaces, but, apart from the results of Armstrong and Singer[5], who showed that citrate is displaced from bone powder *in vitro* by high levels of phosphate, there is little experimental evidence in support of this view. The experiments to be reported in this communication were undertaken with the object of investigating this problem, using some of the bone materials and fractionating techniques which were employed for the study of the magnesium of bone mineral[6]. The experimental procedures were fully described in this paper, and in the present report only the minimum of detail is given.

Two samples of bone material were used in these experiments, normal ox-bone and hen-bone high in magnesium, phosphate and citrate. Both samples were dried, fat-extracted and ground to pass a 300 mesh (B.S.) sieve. To confirm that the citrate of bone is associated with the mineral fraction rather than with the organic matrix, the ox-bone was analysed for citrate before and after removal of the organic matrix by boiling ethylenediamine[7]. As a result of this treatment, the percentage of citric acid in the mineral matter fell from 1.34 to 0.83 while the CO_2 percentage increased from 5.27 to 5.43. The extraction was carried out at 118° and it appears that some of the citrate was converted into carbonate during the treatment. While this experiment did not prove that all the citrate is present in the mineral fraction, it served to show that a substantial part of it at least is associated with this fraction.

Samples of both types of bone (5 g) were then extracted with 12 successive 150-ml portions of either 0.01 N HCl or an 0.01 N solution of the ammonium salt of ethylenediaminetetraacetic acid at pH 7.5 by shaking followed by centrifugation, and after each extraction a sample of the undissolved material was analysed for citric acid by the method of Taylor[8]. The percentage of citrate present in the original bone samples and in the powders remaining undissolved after the successive extractions are given in Table I. The effect of the two extractants was entirely different. Whereas the citrate content of the acid residues increased with each extraction, the EDTA residues became progressively lower in citrate, with the result that the ash of the final residues contained approximately half as much citrate as the ash of the original materials. The amounts of calcium and phosphorus extracted by the EDTA were approximately two-thirds of the amounts extracted by the acid[6]. It thus appears very unlikely that citrate is present in the apatite lattice, but these results are consistent with the view[3,4] that citrate ions are adsorbed on the surfaces of the bone crystals.

During the acid extraction, Mg and CO_2[6], and Na and K[9] were preferentially removed from the bone samples but citrate was retained on the bone powder, and it is clear, therefore, that citrate ions must be very firmly bound to the bone crystals.

Abbreviation: EDTA, ethylenediaminetetraacetic acid.

TABLE I

THE CITRATE CONTENT OF THE RESIDUES FROM THE HCl AND EDTA EXTRACTIONS OF OX AND HEN BONE AND OF THE ORIGINAL MATERIALS, EXPRESSED AS THE PERCENTAGE OF CITRIC ACID IN THE CALCULATED ASH*

No. of extractions	*Acid*		*EDTA*	
	Ox	*Hen*	*Ox*	*Hen*
0	1.34	1.69	1.34	1.69
1	1.36	1.78	1.30	1.69
2	1.33	1.84	1.23	1.56
3	1.41	1.86	1.23	1.47
4	1.39	1.95	1.17	1.39
5	1.40	2.01	1.11	1.28
6	1.50	2.06	1.11	1.24
7	1.44	2.11	0.98	1.12
8	1.51	2.16	1.00	1.06
9	1.60	2.23	0.87	0.99
10	1.71	2.32	0.88	0.88
11	1.79	2.46	0.76	0.81
12	1.83	2.29	0.71	0.70

* Calculated by the method used by TAYLOR AND MOORE[13].

This conclusion is supported by the observations of HARRISON, HARRISON AND PARK[10], who showed that the bone citrate of rats did not fall in response to a fall in blood citric acid and by similar observations in the domestic fowl[11].

In spite of the strong affinity between citrate and bone, EDTA was capable of preferentially removing it from the bone powders, which suggests that the citrate is adsorbed as $(\text{Ca citrate})^-$ rather than $\text{citrate}^{\equiv}$ ions. EDTA would chelate calcium present in this form, liberating citrate ions into solution, but it would not be expected to combine with $\text{citrate}^{\equiv}$ ions. It is thought that the greater part of the citric acid of blood plasma is present in the form of the complex $(\text{Ca citrate})^-$, so that, in view of the known surface properties of bone crystals[12], the presence of this ion in bone might be expected.

Department of Physiological Chemistry, The University,
Reading (Great Britain)
T. G. TAYLOR

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Sedimentation characteristics of bacterial ribonucleoprotein obtained at different periods during the cell-division cycle

A large proportion of the ribonucleic acid of bacteria occurs in the form of ribonucleoprotein particles[1]. In growing *Escherichia coli*, these particles (ribosomes) are distributed among a number of different classes having sedimentation coefficients of approximately 20 S, 30 S, 50 S, 70 S and 100 S. The sedimentation coefficient of the largest particle varies between 70 S and 100 S depending on the magnesium concentration. At low concentration (10^{-4} *M*) the 70-S and 100-S particles, dissociate into 30-S and 50-S particles[2,3]. The 30-S and 50-S particles are observed, however, even when the magnesium concentration is high enough to prevent this dissociation. They appear to be the precursors of the larger particles as they are the first to incorporate radioactive materials[4]. More recently evidence has been obtained that the larger ribosomes provide the principal sites for protein synthesis[5].

Using unsynchronized cultures it is not possible to tell whether the different groups of particles exist at all times in each cell or whether the particles change from one class to another in synchrony with the division cycle of an individual cell. If the second alternative were correct samples taken from a synchronized culture at different times during the division cycle should show marked changes in the proportions of the different particles.

Preliminary experiments showed that the ribosomes derived from an exponentially growing culture of *Alkaligenes fecalis* gave a distribution pattern similar to that obtained from *E. coli*. Also the same dissociation occurred at low magnesium concentration. Accordingly the synchronized cultures of *Alk. fecalis* which were available seemed quite suitable for observation of the ribosome pattern during synchronized division.

A 380-ml synchronized culture of *Alk. fecalis* was obtained by a modification of the filtration method described previously[6]. Synchrony was measured by following viable-colony count and total bacterial-cell count, the latter measured in the Coulter Particle Counter (current setting of 6, threshold 12 maximum gain) on samples taken at intervals and fixed in 0.2 % formalin. The growth pattern of this culture is shown in Fig. 1. 50-ml samples were taken as indicated by arrows 1–7 and dumped onto 20 g

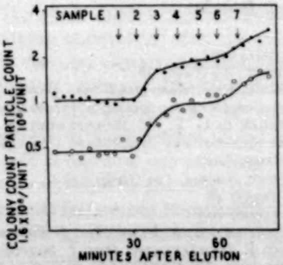

Fig. 1. Synchronous growth of *Alk. fecalis* LB. Bacteria eluted from filter paper used for selection of large cells at zero time. Arrows indicate time of removal of samples for RNA analysis. ○, colony count; ●, particle count.

Abbreviation: RNA, ribonucleic acid.

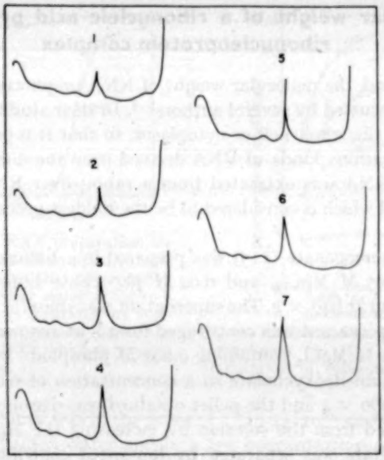

Fig. 2. Analytical ultracentrifuge patterns of *Alk. fecalis*. Samples were taken from a synchronized culture at the times indicated in Fig. 1. The sedimentation coefficient of the largest component is approximately 70 S.

of crushed frozen medium at approximately —70°. The samples were then centrifuged at 2° and washed twice in buffer containing 0.01 *M* tris(hydroxymethyl)aminomethane, 0.004 *M* sodium succinate, 0.01 *M* magnesium acetate pH 7.6. The samples were finally suspended in 0.6 ml of the same buffer and frozen until they could be analyzed. For analysis, the frozen samples were thawed and broken in a modified French pressure cell and immediately run in the model E Spinco ultracentrifuge.

The results are presented in Fig. 2. As may be seen, all of the components were present in the extracts made at different times from the synchronized culture. At no time in the division cycle was one form of ribosome present in quantities strikingly different from those found in exponential cultures. From this it can be concluded that the ribosome pattern which has been studied in exponential cultures represents that of the individual cell and is not merely an average pattern resulting from the summation of different patterns present in cells at different phases of the division cycle.

Department of Terrestrial Magnetism, R. J. Britten
Carnegie Institution of Washington, Washington, D.C., K. G. Lark
Department of Microbiology, F. Norcross
Saint Louis University School of Medicine, St. Louis, Mo. (U.S.A.)

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On the molecular weight of a ribonucleic acid preparation from a ribonucleoprotein complex

The homogeneity and the molecular weight of RNA preparations from mammalian tissues have been discussed by several authors[1–4]. In their studies, however, the RNA was extracted from the whole cell or cytoplasm, so that it is probable that the preparation contained various kinds of RNA derived from the different elements of the cell. In this study RNA was extracted from a rabbit-liver RNP preparation, by a method using phenol which is considered to be the mildest procedure for the preparation of RNA [5,6].

A rabbit-liver homogenate (1:5) was prepared in a buffered medium composed of 0.12 *M* KCl, 0.005 *M* $MgCl_2$, and 0.02 *M* phosphate buffer, pH 7.6, and then centrifuged for 20 min at 640 × *g*. The supernatant was spun for 30 min at 44,000 × *g* and the resulting supernatant was centrifuged for 2 h at 105,000 × *g*. The pellet was resuspended in 0.001 *M* $MgCl_2$ containing 0.002 *M* phosphate buffer. To this suspension was added sodium deoxycholate in a concentration of 0.6–0.8 %. Then it was spun for 2 h at 105,000 × *g* and the pellet obtained was dissolved in 0.001 *M* $MgCl_2$. RNP was precipitated from the solution by increasing the $MgCl_2$ concentration to 0.05 *M*[8]. The precipitate was separated by low-speed centrifugation, washed once with 0.01 *M* $MgCl_2$, resuspended in distilled water, and dialyzed to remove excess Mg^{++}. Contaminating metalloproteins[7] were removed by this procedure. The N:P ratio of the final RNP preparation was about 3.3.

Sedimentation analysis of the RNP preparation gave four peaks at 34–40 S, 50–60 S, 70–80 S, and 100–120 S, of which the main peak was at 70–80 S. However, the quantitative relationship between the four peaks depended upon the medium employed, particularly upon the concentration of bivalent ions such as Mg^{++} and Ca^{++}. When the Mg^{++} concentration was reduced by the use of EDTA, the sedimentation coefficient was 34–40 S (value for infinite dilution: 40 S). When the preparation of RNP was performed with an Mg-free medium, the main peak obtained was at 50–60 S as has been reported previously[7]. Therefore, it is assumed that the components sedimenting faster than 40 S are aggregates of a 40-S unit, in agreement with results obtained with the RNP of rat liver[8]. The molecular weight of the 40-S RNP complex was approximately $1.4 \cdot 10^6$, estimated from viscosity measurements assuming that the partial specific volume was 0.66[9].

The preparation of RNA with phenol was carried out in the manner described by GIERER AND SCHRAMM[5] and KIRBY[6]. The RNP suspension, after addition of EDTA to a concentration of 0.01 *M*, was shaken for 1 h at 40° with an equal volume of 80 % phenol. After centrifugation, the supernatant was separated and the residue was washed twice with water. RNA was precipitated as the sodium salt from the combined aqueous extract, by the addition of ethanol. Reprecipitation was carried out twice. The final solution was dialyzed against distilled water and freeze-dried. The recovery of RNA from the RNP preparation was about 90 %.

The N:P ratio of the RNA preparation was 1.78 and the extinction coefficient per atom P was approximately 8,100 at 258 mμ. Electrophoresis of the preparation gave a single sharp peak when the analysis was carried out at 4° in acetate buffer of

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Abbreviations: RNA, ribonucleic acid; RNP, ribonucleoprotein; EDTA, ethylenediaminetetraacetate.

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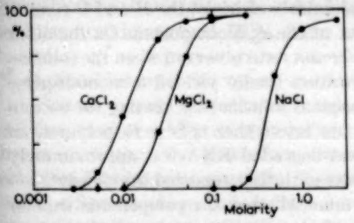

Fig. 1. Precipitation of RNA preparation by salts. To 0.4 % RNA was added an equal volume of a salt solution of double the concentration shown on the abscissa. After standing for 10 h at 0°, the precipitate was separated by low-speed centrifugation. The ordinate shows the percentage of the total RNA which is precipitated.

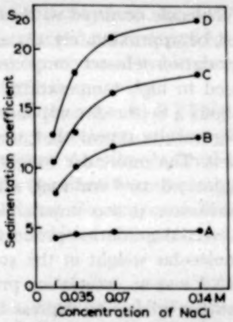

Fig. 2. Sedimentation coefficients of the four components (A, B, C and D) of the RNA preparation in different salt concentrations.

pH 6.0, I 0.1. The mobility was in the order of 13 to $14 \cdot 10^{-5}$ cm^2/sec. V. The preparation was soluble in distilled water and in a low concentration of salt, but a greater part of it became insoluble in a high concentration of salts such as NaCl, MgCl$_2$, and CaCl$_2$ (Fig. 1), as has been observed in RNA's of high molecular weight[10–13]. When subjected to sedimentation analysis in a low NaCl concentration the preparation gave four peaks (called A, B, C, and D from slower to faster). The sedimentation coefficients of these peaks increased with the rise of salt concentration due to a charge effect (Fig. 2). The approximate molecular weight of the faster 23 S component estimated from viscosity measurements to be approx. $6.2 \cdot 10^5$. This is about the value to be expected if this RNA molecule is a component of the 40 S RNP complex which contains about 45 % RNA.

It was noted, however, that the quantitative ratios of the four components depended upon the concentration of salt (Table I). As a rule, peak B was the highest in an NaCl concentration lower than 0.035 *M*. However, the highest peak in 0.14 *M* NaCl was due to the C component and that in 0.2 *M* NaCl to the D component, with corresponding decreases in the B component. The results suggest that association of

TABLE I

QUANTITATIVE RATIOS OF THE FOUR COMPONENTS APPEARING IN THE ANALYSIS OF RNA PREPARATION

The A, B, C, and D components are the same as marked in Fig. 1. The amount of each component was estimated from the area under the sedimentation peak, corrected with respect to sector effect.

NaCl (M)	*Proportion (%) of each component*			
	A (4.3 S)	*B (12.6 S)*	*C (17.8 S)*	*D (23.2 S)*
0.017	14	53	26	7
0.035	13	52	26	8
0.07	12	39	37	12
0.14	10	22	49	18
0.20	10	17	30	43
0.14, treated at 90° for 60 min	100	—	—	—

the RNA molecule occurred with salt. The molecular weights of the B and C components must be approximately 1/3 and 2/3 that of the 23-S component. On the other hand, degradation of faster components to slower units was observed when the solution was exposed to high temperature. The preparation finally yielded a monodisperse peak of about 4 S (the A component of the original solution) by heating for 60 min at 90°. The results reveal that the components faster than 4 S were polymers of the 4-S unit. The molecular weight of the heat-degraded RNA was approximately 30,000 ($D_{20} = 7.8 \cdot 10^{-7}$ cm^2/sec), which coincides with that reported previously[7].

In conclusion, it was impossible to determine whether the components smaller than 23 S were degradation products of the 23-S component, which was the highest possible molecular weight in the 40-S RNP complex, or whether such highly polymerized RNA was an association product of relatively smaller units.

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National Institute of Animal Health, Kokobunji, Tokyo (Japan) M. TAKANAMI

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The cofactor specificity of ethanol dehydrogenase from *Acetobacter peroxydans*

Bacteria of the strictly aerobic genus *Acetobacter* grow readily with ethanol as carbon and energy source; it is thus of interest to compare the ethanol dehydrogenase of these organisms with those of animals and of organisms which produce ethanol as a product of fermentation. KING AND CHELDELIN[1] partially purified the ethanol dehydrogenase of *A. suboxydans*, and found it to be specific for DPN and generally similar to the corresponding enzyme from yeast. ATKINSON[2,3] found cell-free preparations of *A. peroxydans* (ATCC strain 838) to contain a DPN-specific ethanol dehydrogenase and a very active DPNH oxidase system that was easily separated from the dehydrogenase by centrifugation. Simultaneously, however, TANENBAUM[4], on the basis of observations on cell-free preparations of NCIB strain 8618, reported that

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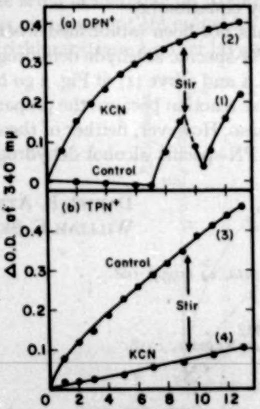
Abbreviations: DPN, diphosphopyridine nucleotide (DPN$^+$, DPNH, oxidized and reduced forms; TPN, triphosphopyridine nucleotide (TPN$^+$, TPNH, oxidized and reduced forms).

the ethanol and acetaldehyde dehydrogenases of *A. peroxydans* are TPN-specific with no activity toward DPN. Since an unusual cofactor requirement for ethanol oxidation in an organism grown on ethanol would be of considerable comparative biochemical interest, this report was further investigated.

SERAT[5] showed that ethanol is indeed oxidized at the expense of TPN$^+$ in crude cell-free extracts. After centrifugation at an average acceleration of 105,000 × *g*, however, neither the precipitate nor the supernatant fraction catalyzed such a reaction. The activity was fully regained when the fractions were recombined, but not if either had been boiled. It thus seemed clear that the supposed TPN-specific alcohol dehydrogenase activity resulted from the combined action of two or more enzymes.

The results to be reported, which confirm this conclusion, were obtained with ATCC strain 838, but experiments with NCIB strain 8618 (ATCC 12874) have given qualitatively identical results. Cells were grown in liquid medium[2] with vigorous aeration, harvested by centrifugation, and disrupted in a modified Hughes press while frozen. The resulting suspension, diluted with phosphate buffer, pH 7.0, to five times the volume of the wet cells and centrifuged at about 2000 × *g* for 20 min, is termed the crude cell-free preparation.

In such preparations, the production of DPNH as a result of ethanol oxidation is not initially apparent because the particulate oxidase system catalyzes immediate reoxidation of the cofactor. Only after exhaustion of dissolved O_2 can the reduction of DPN$^+$ be observed[3]. When the oxidase is poisoned with cyanide, the dehydrogenase reaction can be followed from the start (Fig. 1a). In striking contrast to these results, the reduction of TPN$^+$ is observed from the moment of enzyme addition, and is strongly inhibited by cyanide (Fig. 1b). Curves (1) and (3) of Fig. 1 confirm TANENBAUM's results[4] and, considered by themselves over the first 7 min, would appear to



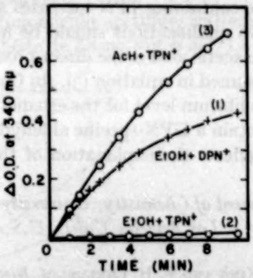


Fig. 2. Cofactor reduction by ethanol and acetaldehyde dehydrogenases in a soluble preparation from *A. peroxydans*. Each cuvette contained 0.05 ml of the supernatant fraction from centrifugation at 105,000 × *g* for 40 min, 0.5 mg DPN$^+$ or TPN$^+$, 100 μmoles ethanol or acetaldehyde, and 100 μmoles phosphate buffer, pH 7.0, in a total vol. of 3.0 ml.

Fig. 1. Oxidation of ethanol at the expense of DPN$^+$ or TPN$^+$ by a crude cell-free preparation of *A. peroxydans*. Each cuvette contained 0.1 ml of the preparation, 100 μmoles ethanol, 0.5 mg DPN$^+$ or TPN$^+$, and 100 μmoles phosphate buffer, pH 7.0, in a total vol. of 3.0 ml. KCN, where present, $1.6 \cdot 10^{-3}$ *M*. Stirred (to introduce oxygen) at time indicated by arrows.

justify his conclusion that the dehydrogenase is specific for TPN. (The lag time before net DPN reduction can be observed is longer when lower concentrations of ethanol or of enzyme are used.)

After removal of the particulate oxidase system by centrifugation, ethanol dehydrogenase is seen to be specific for DPN (Fig. 2). The supernatant fraction also contains the TPN-specific aldehyde dehydrogenase reported by TANENBAUM[4] (Fig. 2). The apparent TPN-specific ethanol dehydrogenase of crude cell-free systems is thus readily explained as resulting from the simultaneous action of ethanol and acetaldehyde dehydrogenases and the DPNH oxidase system:

$$CH_3CH_2OH + DPN^+ \rightleftharpoons CH_3CHO + DPNH + H^+ \text{ (supernatant)} \quad (1)$$

$$DPNH + \tfrac{1}{2}O_2 + H^+ \rightarrow DPN^+ + H_2O \text{ (precipitate)} \quad (2)$$

$$CH_3CHO + TPN^+ + H_2O \rightleftharpoons CH_3COOH + TPNH + H^+ \text{ (supernatant)} \quad (3)$$

$$\text{Sum: } CH_3CH_2OH + TPN^+ + \tfrac{1}{2}O_2 \rightarrow CH_3COOH + TPNH + H^+ \quad (4)$$

Cyclic oxidation and reduction of endogenous DPN thus permits the reduction of added TPN+ and mimics a TPN-specific ethanol dehydrogenase. Cyanide inhibition of the net reaction follows from its effect on the oxidase system (eqn. 2).

When the DPNH produced by ethanol dehydrogenase in a crude preparation begins to accumulate after the exhaustion of dissolved O_2, the addition of O_2 (as by stirring) causes a decrease in absorbancy (Fig. 1, curve 1). No such effect is observed in the case of TPNH, since our cell-free preparations lack TPNH oxidase activity.

When air is stirred into reaction mixtures of the types used for curves (1) and (3) of Fig. 1 after apparent equilibrium has been reached (at a later time than is shown on the figure) the expected results are obtained: the absorbancy of the reaction mixture with added DPN+ decreases because of DPNH oxidation, while that of the mixture containing TPN increases as predicted by equation (4).

Two qualifications should be noted: (a) It has not been established whether or not free acetic acid is the direct product of the TPN-specific aldehyde dehydrogenase, as is assumed in equation (3). (b) Curve (2) of Fig. 1 and curve (1) of Fig. 2 go beyond the equilibrium level for the ethanol dehydrogenase reaction because the preparations also contain a DPN-specific aldehyde dehydrogenase. However, neither of these comments affects the explanation of the apparent TPN-specific alcohol dehydrogenase.

Department of Chemistry, University of California,
Los Angeles, Calif. (U.S.A.)

DANIEL E. ATKINSON
WILLIAM F. SERAT*

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* Present address: Rohm and Haas Co., San Francisco, Calif. (U.S.A.).

Incorporation de la [^{14}C]valine dans les protéines des fractions subcellulaires de réticulocytes de lapin*

Les réticulocytes de lapin sont capables de réaliser une synthèse d'hémoglobine *in vitro*. Les travaux de DINTZIS ET BORSOOK[1] sur les réticulocytes entiers, de SCHWEET *et al.*[2] sur les préparations acellulaires de réticulocytes ont montré le rôle fondamental des microsomes dans la synthèse de l'hémoglobine. Récemment, RABINOWITZ ET OLSON[3] ont étudié, dans un travail qui ne nous est parvenu qu'après l'achèvement de celui-ci, l'incorporation des acides aminés dans les fractions protéiques des réticulocytes. Les conclusions auxquelles ils aboutissent sont en partie analogues aux nôtres.

Les réticulocytes ont été obtenus par ponction cardiaque de lapin ayant reçu 2 injections de chlorhydrate de phénylhydrazine; ils ont été incubés à 37°, en présence de citrate de fer ammoniacal, de $NaHCO_3$, d'un mélange d'acides aminés formé à la concentration finale de 0.2 m*M* et de [^{14}C]valine à la concentration de 10 mC pour les globules rouges obtenus à partir de 100 ml de sang, dans un incubateur de Dubnoff, sous courant gazeux d'un mélange comprenant 95 % de O_2 et 5 % de CO_2, selon la technique décrite précédemment[4], la durée des incubations variant de 1 à 60 min. Après incubation, les globules rouges lavés sont hémolysés par 4 volumes de $MgCl_2$ 0.005 *M*, puis additionnés de 1 volume de saccharose 1.5 *M* et KCl 0.15 *M*, selon la technique de SCHWEET *et al.*[2].

On sépare les fractions suivantes: (a) Sédiment de centrifugation à 1000 × *g* pendant 1 min. (b) Sédiment de centrifugation du surnageant précédent à 10,000 × *g* pendant 10 min. (c) Sédiment du surnageant précédent dans la centrifugeuse Spinco préparative, 60 min à 105,000 × *g*. Cette fraction est formée de microsomes. (d) "Enzyme pH 5" obtenu par centrifugation du surnageant précédent après addition d'acide acétique 1 *N* jusqu'à obtention du pH 5.15. (e) Hémoglobine pure obtenue selon la méthode de ROCHE *et al.*[5]. par relargage.

Les protéines ont été préparées par précipitation trichloracétique, traitement par acide trichloracétique à 90° et lavage selon la méthode de ZAMECNIK *et al.*[6]. La mesure

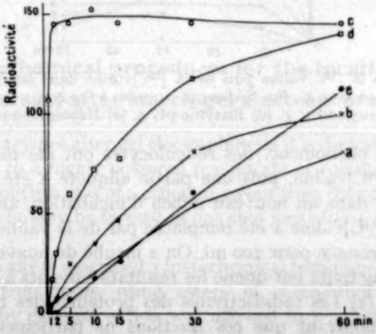

Fig. 1. Incorporation de la [^{14}C]valine dans les fractions réticulocytaires. (a) Sédiment 1 min à 1000 × *g*. (b) Sédiment 10 min à 10,000 × *g*. (c) Sédiment 60 min à 105,000 × *g*. (d) Enzyme pH 5. (e) Hémoglobine.

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* Avec la collaboration technique de L. TIKHONITZKY Laboratoire de Recherches de Biochimie Médicale, Hôpital des Enfants-Malades, Paris (France).

de radioactivité a été faite au compteur de Geiger Müller en utilisant des cupules de 0.28 cm^2 de surface, les corrections d'épaisseur ont été effectuées.

Les résultats suivants ont été obtenus: (Fig. 1)

1. Les fractions (a), (b), (d) ont incorporé la [^{14}C]valine dès la 1ère minute, l'incorporation est linéaire pendant 30 min pour les fractions (a) et (b), puis la courbe tend à s'aplatir; l'incorporation est beaucoup plus intense dans l'enzyme pH 5.

2. Les microsomes incorporent très rapidement l'acide aminé radioactif, l'incorporation est maxima en 2 min et la radioactivité reste constante jusqu'à la 60 ème minute. Ce résultat témoigne en particulier d'une pénétration très rapide de la valine à l'intérieur des réticulocytes.

3. L'incorporation dans l'hémoglobine présente un temps de latence de 2 min suivi d'une incorporation linéaire jusqu'à la 60 ème minute.

La constance de la radioactivité des protéines des microsomes peut s'expliquer soit par une perte rapide, dans les conditions expérimentales, de l'activité métabolique de ces particules, soit par la participation à l'équilibre dynamique. Dans ce dernier cas, cette participation peut se traduire soit par des échanges entre acides aminés libres et incorporés, soit par une synthèse et secrétion active de protéines.

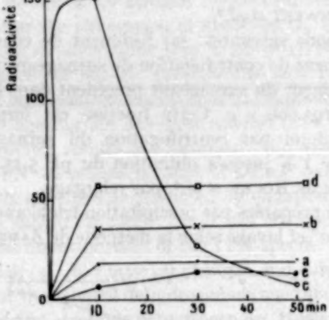

Fig. 2. Incorporation de la [^{14}C]valine puis de la [^{12}C]valine dans les fractions réticulocytaires. (a) Sédiment 1 min à 1000 × *g*. (b) Sédiment 10 min à 10,000 × *g*. (c) Sédiment 60 min à 105,000 × *g*. (d) Enzyme pH 5. (e) Hémoglobine.

Pour étudier ce phénomène, des réticulocytes ont été mis à incuber pendant 10 min avec de la [^{14}C]valine, puis une partie aliquote a été centrifugée, lavée et remise en suspension dans un nouveau milieu d'incubation, analogue au précédent, mais dans lequel la [^{14}C]valine a été remplacée par de la valine non radioactive à la concentration de 0.1 mmole pour 100 ml. On a incubé de nouveau 20 min et 40 min. Les mesures de radioactivité ont donné les résultats suivants à la suite des dernières incubations (Fig. 2). (1) Les radioactivités des protéines des fractions (a), (b), (d), restent constantes, montrant que ces fractions ne participent pas activement à l'équilibre dynamique. (2) La radioactivité des protéines des microsomes décroît très rapidement. (3) La radioactivité de l'hémoglobine augmente légèrement, mais significativement pendant les premières 20 min de l'incubation avec la valine non radioactive.

Ces résultats peuvent s'interpréter en admettant que l'hémoglobine est synthétisée dans les microsomes, puis libérée dans la partie soluble de la cellule. La très faible quantité des microsomes par rapport à la grande quantité d'hémoglobine explique dans cette hypothèse qu'une forte chute de la radioactivité des microsomes ne corresponde qu'à une faible augmentation de la radioactivité de l'hémoglobine. Le délai de 2 min avant l'incorporation de la [^{14}C]valine dans l'hémoglobine n'a pas été trouvé par RABINOWITZ ET OLSON[3]. BORSOOK[7] a calculé une durée de synthèse de l'hémoglobine de 100 min en admettant un poids moléculaire de la matrice nucléoprotéique de 60,000, mais LOPTFIELD ET EIGNER[8] ont ramené cette durée à 3 min en admettant que ce poids moléculaire est de 2,000,000, valeur actuellement plus vraisemblable.

Nous remercions l'Institut National d'Hygiène, la Caisse Nationale de Sécurité Sociale et "The National Institute of Arthritis and Metabolic Diseases, Public Health Service" (Subvention de Recherches No. A. 2773), de l'aide apportée à ce travail.

Laboratoire de Recherches de Biochimie Médicale,
Hôpital des Enfants-Malades, Paris (France)

JACQUES KRUH
GEORGES SCHAPIRA
JEAN-CLAUDE DREYFUS

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Histochemical procedures for the location of esterases after starch-gel electrophoresis

For the detection of enzymes after gel electrophoresis it is desirable that the technique used should give rise to a highly coloured precipitate or solution after reacting with a substrate that is colourless or is of a different colour from the product of the reaction. The colour should preferably be formed in one step and after a short period of action at room temperature.

HUNTER AND MARKET[1] described a histochemical technique of detecting esterases after starch-gel electrophoresis, using esters of α-naphthol as substrates. The α-naphthol released by esterase action was allowed to react with diazo compounds to give rise to highly coloured complexes according to the technique of GOMORI[2]. This technique has not given satisfactory results in our hands.

Recently, KRAMER AND GAMSON[3] described the use of indophenol acetate as a

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Abbreviation: Tris, tris(hydroxymethyl)aminomethane.

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substrate for the determination of acetylcholine esterase. This compound has a yellowish colour in buffer solution at pH 8.0 and after esterase action liberates the intensely blue-coloured indophenol. This substrate has been used by MASTER AND RAO[4] for the detection of cholinesterase in cobra venom after electrophoretic separation on starch gel.

In the present communication chromogenic substrates based on esters of 2,6-dichlorophenolindophenol are described for the detection of aliesterases and lipase after electrophoretic separation on starch gel.

Electrophoresis of cobra venom (the source of the esterase) was carried out on starch gel according to the method of SMITHIES[5] using a discontinuous system of buffers as recommended by POULIK[6].

Esters of 2,6-dichlorophenolindophenol were prepared by allowing the sodium salt of the dye to react with the anhydrides of acetic acid, butyric acid and caproic acid at room temperature (28–30°) overnight and then following the usual procedure for acylation. It has been found that the acetyl, butyryl and caproyl esters of 2,6-dichlorophenolindophenol are dark brown in colour and after hydrolysis give back the intensely coloured blue-purple 2,6-dichlorophenolindophenol. 0.25 ml of 2.5 % ethanolic solution of these derivatives was diluted to 2.5 ml with Tris–citrate buffer pH 8.6 immediately before use.

One of the strips was stained for proteins with Amido Black 10B and others were covered with a layer of the ester solutions and left at room temperature (25°). Within about 15 min the zones of the esterase activity started appearing and within 60 min were dark enough to be photographed. By comparing the strips stained for proteins and those treated with esters the position of the esterase could be located.

In the case of cobra venom the esterase activity with acetyl, butyryl and caproyl derivatives were located at the same position corresponding to the fastest-moving component on the anode side.

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Department of Antitoxins and Sera, Haffkine Institute, Parel, Bombay (India) R. W. P. MASTER

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Preparation of N-acetylneuraminic acid from N-acetyl-D-mannosamine

N-Acetylneuraminic acid was synthesized[1], in 1–2 % yield from N-acetyl-D-glucosamine and oxaloacetate at pH 11. Subsequently, Comb and Roseman[2] showed that an enzyme of *Clostridium perfringens* catalyses the reaction.

N-acetylneuraminic acid $\rightleftharpoons$ N-acetyl-D-mannosamine + pyruvate, but will not utilize N-acetyl-D-glucosamine; and it was also shown[3,4] that N-acetyl-D-glucosamine at pH 11 is partially epimerized to an equilibrium mixture containing N-acetyl-D-mannosamine. We therefore tried the condensation at pH 11 of N-acetyl-D-mannosamine with oxaloacetate. Brug and Paerels reported[5], while our work was in progress, that this condensation gave yields no better than 1–2 %, though variations of procedure were tried; we found, however, that a yield of 8–11 % was attainable by slight modifications of the original method. Further evidence is thus added to that already available[2–4,7] that N-acetylneuraminic acid is related stereochemically to mannosamine, not glucosamine.

Our experiments, though not numerous enough to define optimum conditions for the condensation, are thought to indicate a practical method for preparing N-acetylneuraminic acid in quantity. Work on this problem has now been discontinued and the results are reported in order to encourage further experiment.

Separation of N-acetyl-D-mannosamine formed by alkaline epimerization of N-acetyl-D-glucosamine had previously been achieved by chromatography on borate-treated paper[3], or preparatively by using *Escherichia coli* to remove residual N-acetyl-D-glucosamine[6]; but a satisfactory separation can be based on differential solubilities. In three experiments, N-acetyl-D-glucosamine (240 g in all) was epimerized (pH 11, 2 days) and the product crystallized from water–ethanol. After separation of N-acetyl-D-glucosamine (158 g), crops of a crystalline mixture (67 g) were obtained. This material was twice stirred (intermittently during 5 min) in a blendor with cold absolute ethanol (23 ml/g); undissolved N-acetyl-D-glucosamine (approx. 30 g in all) was removed, and the filtrate was concentrated (after addition of water) to a small volume. On addition of ethanol, N-acetyl-D-mannosamine monohydrate crystallized, 30 g in all being finally obtained. This was chromatographically homogeneous and its composition, behaviour on melting, and mutarotation in aqueous solution agreed with published reports[6,8,9].

Oxaloacetic acid (2.25 g) in water (16 ml) was brought to pH 9 by 10 *N* NaOH. N-Acetyl-D-mannosamine monohydrate (4.08 g) was added and the pH was raised to 11.0 and maintained at this level. Oxaloacetic acid (0.56 g) was added after 3 and after 6 h. The quantitative Bial assay reached a maximum (equivalent to a yield of 40–42 % N-acetylneuraminic acid) after 7–9 h; it then declined to reach a steady value (29 % "yield") at 12 h. Assay, after removal of Na^+, by chromatography on paper (butanol–pyridine–water (6 : 4 : 3, v/v/v)) appeared to indicate maximal formation of N-acetylneuraminic acid after 24 h, but poor separation of spots precluded an accurate estimate.

Isolation of N-acetylneuraminic acid from the reaction mixture followed the earlier procedure[1], except for the final chromatography. "Activated, acid-washed, decolorizing charcoal powder" (British Drug Houses Ltd.) was shaken in aqueous suspension on a 280-mesh sieve and the material retained was stored under water until required, when a column 40 × 200 mm was prepared and washed with 0.3 *N* formic acid and with water. Chromatography then proceeded as previously described; those fractions which were Ehrlich-positive (22-ml fractions; serial numbers around 60–100) were separately evaporated *in vacuo*. The fractions showing spontaneous crystallization were pooled and recrystallized by evaporating water to small bulk and adding acetic acid (2 vol.). After 3 days at 5–10° the crystals were collected and recrystallized[1] from the Blix solvent system. The product was chromatographically homogeneous and its infrared spectrum was identical with that of authentic N-acetylneuraminic acid. The two experiments worked up in this manner are summarized in Table I.

TABLE I

PREPARATION OF N-ACETYLNEURAMINIC ACID

Expt. No.	*Reaction time (h)*	*pH*	*Product (mg) after 1st recrystallization*	*Product (mg) after 2nd recrystallization*	*Yield %*
4	24	11.7 during first hour, then 11.0	713	607	11.5
6	24	11.0	527	425	8

In two other experiments a smaller carbon column (40 × 100 mm) was used; the products of the first crystallization were not suitable for recrystallization in the Blix system and a second recrystallization from acetic acid (83 %) was used instead. In one experiment (9 h at pH 11.0) the final yield was 524 mg (10 %) and in another (24 h at pH 11.0), 474 mg (9 %).

Human Nutrition Research Unit, Medical Research Council, PATRICIA M. CARROLL
National Institute for Medical Research, London (Great Britain) J. W. CORNFORTH

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Temperature coefficients of the respiratory unit

As shown in previous communications[1] the respiratory unit may be figured as a spatially organized system the reactivity of which is regulated by structural arrangements of the participating enzymes and co-factors. A new spectrophotometric technique enabled determination of start gaps and apparent reaction velocities of the participating enzymes[2]. Each respiratory enzyme displays a bilateral activity, *viz.* its multimolecular body receives electrons at one place and emits them at another.

In the steady state of the intact system both reactions are running with equal speed and the respiratory unit behaves as a mechanism transferring electrons between metabolites and oxygen. Previous communications showed the effect of O_2,N_2, antimycin A, cyanide etc. on the apparent velocities of reduction and reoxidation[2]. The effect of an increase or a retardation of the on- and off-reactions of the respiratory unit was also studied. An acceleration of the on- reaction of the total system is brought about by adding glucose to starved baker's yeast, and the off-reaction $a_3 \xrightarrow{e} O_2$ is influenced by regulation of the O_2 pressure or by addition of low concentrations of HCN or CO[3]. In a series of new experiments the balance between the on- and off- reactions of the respiratory unit was changed by raising or lowering the temperature.

Using a previously described technique[2] suspensions of 10% washed baker's yeast were alternately gassed with N_2 or air. The reactions of cytochromes *c* and *b* were measured by means of continuous spectrophotometric recording of the band tops, or by rapid oscillographic recording of the spectral portion 540–570 mμ. The quartz recipient was enclosed in a water bath, the temperatures of which was varied between 5° and 50°.

Fig. 1 shows the apparent reaction velocities at different temperatures. Between 5° and about 35°–40°, the velocity of reduction (in N_2) follows the course usual for a chemical reaction, with a Q_{10} value of 2.0. Above an optimum at 40°–45° the curves sharply bend downwards, probably due to damage to the protoplasm.

The velocity of reoxidation (in air) is a reversed image of that of reduction, starting with highest values at 5° and attaining a minimum at about 45°.

According to an idea developed in previous communications[1] the flux of electrons through the respiratory unit is similar to that through a metal of low conductivity or rather a semi-conductor, viz. the electrons follow structural tracks which are interspaced by non-conductive material. It must accordingly be expected that the temperature exerts a fairly uniform influence on the interaction between the single

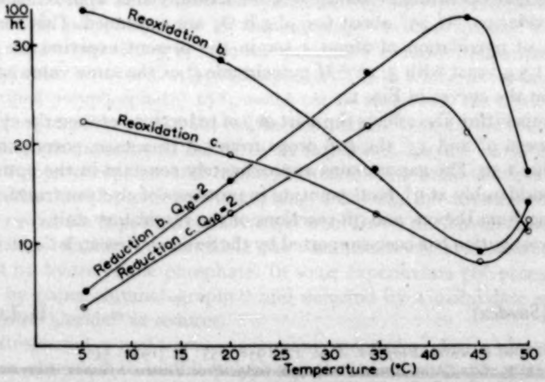

Fig. 1. Effect of temperature on the reduction (in N_2) and reoxidation (in air) of cytochromes *c* and *b* in washed baker's yeast (10%) suspension. Recipient, 19 mm wide circulation vessels[3]. Automatic alternation at constant intervals between N_2 and air. The values are quoted as 100/ht = apparent velocity; ht = half time (sec) of full reaction.

enzymes and factors, as shown by the parallell course of the reaction curves of cytochromes *c* and *b*. It is reasonable to assume that the temperature primarily regulates the on- and off-reactions of the respiratory unit, viz. the dehydrogenation of the metabolites and the final oxidation, with less effect on the internal speed of electron transference (see below).

The time course of reduction and reoxidation are both dependent on the mutual velocity of the on- and off-reactions of the respiratory unit. It was previously shown that cytochrome a_3 starts reduction as soon as the O_2 pressure has attained a certain minimum. The course of reduction is then a compromise between oxidation by the last traces of external O_2 and the internal supply of electrons from the on-reaction of the respiratory unit. The "normal" Q_{10} value of the process of reduction points to the conclusion that the last named process is the dominating one.

Owing to the rapid penetration of O_2 through the cell membranes the time course of reoxidation is largely determined by the internal supply of electrons, which delays the oxidation. As a consequence, a lowering of the temperature raises the speed of reoxidation, as shown in Fig. 1. The rise is, however, slowed down by the response of the over-all reaction $a_3 \xrightarrow{e} O_2$ to temperature. Because the supply of electrons decreases four times with a drop from 25° to 5° the reoxidation at 5° would be expected to rise 60–70 % more than observed, provided that the off-reaction were insensitive to the temperature, which is obviously not the case. The calculation of Q_{10} of reoxidation encounters difficulties but an extrapolation of the curve near to the minimum points to a value of about 2. A number of physical processes, *e.g.* diffusion and adsorption of O_2, are here rate-limiting factors, but they are characterized by low Q_{10} values. Temperature factors of permeability (to O_2) through living membranes may on the other hand be higher. Intermediates may also be involved in the process of reduction of O_2.

The measured velocity of reoxidation agrees with the observed intensity of respiration of yeast supplied with 1–2 % glucose in the absence of salts. The cytochromes are here operating at about 90 % reduction, viz. at approximately maximal power of oxidation. At 25° about 600 µl/g/h O_2 are consumed. This corresponds to a half-time of reoxidation of about 1 sec in the present experiments (each vessel containing 1.5 g yeast with $3 \cdot 10^{-8}$ *M* cytochrome *c*) or the same value as that extrapolated from the curves in Fig. 1.

The temperature also affects the start gap of reduction between the cytochromes *c* and *b*. Between 9° and 23° the gap drops from 5.5 to 2.5 sec, corresponding to the low Q_{10}-value 1.59. The gap remains approximately constant in the optimum region but rises considerably at 5°, facts pointing to processes of electron transference which are deviating from the on- and off-reactions of the respiratory unit.

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Penningby (*Sweden*) H. LUNDEGÅRDH

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Enzymic formation of polyphosphate in an anaerobic bacterium

The presence of inorganic polyphosphate in microorganisms[1-3] and lower animals[4,5] has been reported by many investigators. Although some information is now available concerning the mechanism of the formation of polyphosphates and their metabolic relation to the adenine nucleotides[6,7], the role of polyphosphates in the cell still remains obscure. It has been observed[8] that extensive synthesis of polyphosphate occurs under aerobic conditions.

In the course of studies on the degradation of creatinine by anaerobic bacteria[9,10] a new strain of *Clostridium** capable of utilizing creatinine as the sole nitrogen and energy source for growth, and CO_2 as the major carbon source, was isolated by the enrichment-culture technique. The growth medium and growth conditions were the same as described in a previous publication[9], except that malt extract was omitted. It was observed that growing cells, or cell suspensions of *Clostridium Ls.* produce 1 mole sarcosine, 2 moles NH_3 and 1 mole CO_2/mole of creatinine degraded.

Cell-free extracts were prepared by submitting cell suspensions from the log-phase of growth to sonic vibration for 10 min at 3–4°. Balance studies with these extracts, as shown in Table I, have demonstrated that creatine is a product of the degradation of creatinine. The results indicate also that the anaerobic dissimilation of creatinine is associated with the esterification of orthophosphate. Approximately 0.2 to 0.6 μmole of orthophosphate was esterified /μmole of creatinine degraded. The P disappearance reached a maximum after 60–80 min incubation at 37°. The subsequent increase in orthophosphate in the incubation mixture may have been due to the action of specific phosphatases present in the extract. When creatinine was omitted from the system, as shown in Table I, a great decrease in P uptake occurred.

Experiments with [^{32}P]orthophosphate have shown that ^{32}P was incorporated into inorganic polyphosphates. The labeled polyphosphates were primarily characterized by their lack of u.v. absorption and by the fact that after hydrolysis for 15 min in *N* HCl at 100° they were converted quantitatively to [^{32}P]orthophosphate. The resolution of the polyphosphates by ion-exchange chromatography is shown in Table II. It can be seen that 37.5 % of the inorganic ^{32}P which disappeared from the incubation mixture was found as pyrophosphate, 23 % as tripolyphosphate and 22 % as non-identified polyphosphate; 18 % could not be recovered. The relative amounts of the polyphosphates formed in the incubation mixture varied from one experiment to another. In some experiments the ^{32}P in the incubation mixture was almost entirely assimilated as pyrophosphate and tripolyphosphate. The isolated tripolyphosphate was further identified by rechromatographing, following the addition of 10 μmoles of synthetic carrier Na tripolyphosphate (a gift from Dr. S. R. KORNBERG) as described in Table II. The mixture was eluted in the same fractions, as measured by radioactivity and by hydrolysable phosphate. In some experiments polyphosphates were determined by paper chromatography[11] and detected by a molybdate spray[12] with benzidine hydrochloride[13] as reducer.

2,4-dinitrophenol, a well known uncoupling agent of oxidative phosphorylation, has also been observed[8] to inhibit polyphosphate formation in yeast under aerobic conditions. In the present investigations, $2 \cdot 10^{-3}$ *M* 2,4-dinitrophenol was found

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* For the purpose of this paper this strain is referred to as *Clostridium Ls.* (Louisiana Strain).

TABLE I

BALANCE STUDY OF CREATININE DEGRADATION BY A DIALYZED CELL-FREE EXTRACT OF *Clostridium Ls.*

The complete system contained the following components in a final vol. of 1 ml: dialyzed cell-free extract containing 5 mg protein*, 100 μmoles of dimethylglutarate buffer pH 7.4, 20 μmoles of [1-^{14}C]creatinine** (5000 counts/min), 10 μmoles K_2HPO_4. Each sample was flushed for 1 min with H_2 and sealed with a rubber stopper. The samples were incubated at 37°. At intervals, 0.1-ml aliquots were removed, added to an equal volume of 6% trichloroacetic acid at 0° and centrifuged. The supernatant was assayed for creatinine, creatine, NH_3, and orthophosphate. For CO_2 estimations, 0.2-ml aliquots were removed from duplicate incubation tubes and added to a tube chilled in an ice bath and containing 0.1 ml 0.2 *N* NaOH. The CO_2 content was estimated in a Warburg apparatus by the conventional method. [1-^{14}C]sarcosine was separated from the incubation mixture by paper chromatography, using *tert.*-butanol–acetic acid–water (2:1:1) as solvent and 1% Na nitroprusside in 10% (v/v) acetaldehyde as spray reagent. A duplicate spot was eluted and counted for radioactivity and the sarcosine concentration thus determined***. Values are expressed as μmoles/ml incubation mixture.

Incubation time (min)	*Creatinine disappeared*	*Products formed*				*P uptake*	
		Creatine	*Sarcosine*	*NH_3*	*CO_2*	*Creatinine present*	*Creatinine absent*
20	2.1	0.8	—	2.65	—	1.70	1.65
40	4.6	0.7	3.5	7.40	—	3.70	1.20
60	7.2	1.2	5.3	10.30	5.10	6.05	1.60
80	10.2	2.3	7.0	15.50	6.95	8.20	2.10
100	12.2	2.9	8.7	16.30	8.70	5.40	2.10
120	14.8	3.4	10.6	22.10	—	3.3	2.4

* Extracts containing 20–25 mg of protein were dialyzed for 3 h at 4° against 3 l 0.01 *M* dimethylglutarate buffer, pH 7.4, containing 0.03% $Na_2S \cdot 9H_2O$, followed by a second dialysis against 1 l distilled water for 1 h.

** [1-^{14}C]creatinine was prepared by autoclaving [1-^{14}C]creatine in 1 *N* HCl for 1 h at 120°.

*** The recovery of [1-^{14}C]sarcosine from the paper chromatogram was over 85%; in determining the sarcosine concentration it was assumed to have the same specific activity as the [1-^{14}C]-creatinine added to the incubation mixture.

TABLE II

RESOLUTION OF THE ^{32}P-CONTAINING PRODUCTS BY ION-EXCHANGE CHROMATOGRAPHY

An incubation mixture as described in Table I but containing unlabeled creatinine and [^{32}P]-orthophosphate was incubated for 80 min at 37°. The reaction was stopped as described in Table I and the entire supernatant containing as reaction products 8 μmoles polyphosphates with 177,000 counts/min was treated with 1 ml acid-washed Norit (20%). After removal of the Norit*, 88–90% of the polyphosphate remained in the solution. The supernatant was then passed through a Dowex-I-Cl resin column (100–200 mesh: 1 × 3 cm) and the polyphosphates eluted with 0.02 *N* HCl–0.1 *N* KCl at a flow rate of 0.6 ml/min. Fractions of 6 ml were collected in the cold by means of an automatic fraction collector. Total phosphate, hydrolysable phosphate (in 1 *N* HCl for 15 min at 100°) and radioactivity were determined in each fraction. The nature of the polyphosphates eluted was determined by comparison with a standard elution pattern of commercial ortho-, pyro-, trimeta-, tetrameta- and hexameta-phosphates in known concentrations, chromatographed and eluted under the same conditions.

	Total P		*Hydrolysable P*		*counts/min*	*^{32}P %*
	μmoles	*%*	*μmoles*	*%*		
Pyrophosphate zone	2.45	30.5	2.37	29.6	66,700	37.5
Tripolyphosphate zone	1.70	21.3	1.68	21.0	40,000	23.0
Non-identified	2.05	26.2	2.09	26.1	39,000	22.0
Not recovered	—	—	—	—	31,000	17.8

* Norit removal was facilitated by addition of 0.3 ml plasma albumin (6 mg/ml)[14].

to inhibit polyphosphate formation under anaerobic conditions in extracts of *Clostridium Ls*.

Studies on the metabolic relationship between the polyphosphates and adenine nucleotides in *Clostridium Ls.* extracts will be reported in a separate paper.

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National Institutes of Health,
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Bethesda, Md. (U.S.A.)

JEKISIEL SZULMAJSTER*
RITA C. GARDINER

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* Present address: Institut de Biologie Physicochimique, 13 rue Pierre Curie, Parie 5ème France.

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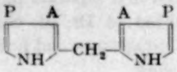
Dipyrromethanes and the biosynthesis of porphyrins

It has been known for some time that the substituted pyrrole porphobilinogen is an intermediate in the biosynthesis of porphyrins[1,2]. An enzyme system present in animals, plants and microorganisms, and called "PBG-deaminase", converts PBG to uroporphyrinogen I[3–5]. This enzyme system has been partially purified so that uroporphyrinogen I and ammonia are the only detectable reaction products. Many crude preparations convert PBG to uroporphyrin III, but when these preparations are preheated to 50–60° then uroporphyrin I is formed[6–8]. These results suggest that at least two enzyme steps are involved in the formation of uroporphyrin III. This is further supported by the experiments of BOGORAD[8] on a "uroporphyrinogen isomerase" from wheat germ. The exact mechanism whereby the cyclic porphyrinogens are formed from PBG is still obscure and no intermediate steps have been detected.

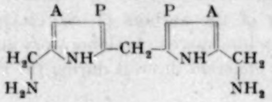
Abbreviation: PBG, porphobilinogen.

MacDonald[9] has described the synthesis of a $\beta\beta'$ substituted dipyrromethane (I) which might be considered a possible intermediate in uroporphyrinogen formation from porphobilinogen.

(I)



(IV)



P = $-CH_2 \cdot CH_2 \cdot COOH$; A = $-CH_2 \cdot COOH$

β-substituents in compounds: (I) PA-AP, (II) AP-AP, (III) AP-PA. Compound (I), the two analogues (II) and (III), and a further derivative (IV) containing aminomethyl substituents in the $\alpha\alpha'$ positions (gifts to Professor C. Rimington from Dr. S. F. MacDonald) have now been tested with crude and partially purified porphyrin-forming enzyme systems derived from *Rhodopseudomonas spheroides*.

The crude enzyme preparation consisted of acetone-dried cells of *R. spheroides* grown semi-anaerobically in the light as previously described[10]. This crude system forms mainly coproporphyrin III from PBG. The partially purified preparation was a PBG-deaminase forming uroporphyrinogen I from PBG; this was prepared as described by Hoare and Heath[5].

With crude enzyme preparations there was no formation of porphyrins when compounds (I), (II) or (III) (100 μg) were incubated with acetone-dried cells of *R. spheroides* (20 mg) at 37°, pH 6.5 or pH 7.6, for periods of up to 4 h. By contrast compound (IV) was converted to uroporphyrin and coproporphyrin (Table I). There was some uroporphyrin formation in the non-enzymic control, but the total porphyrin formation in the presence of the crude enzyme was always greater than that in the control. Paper chromatography of the coproporphyrin formed[11] revealed the presence of coproporphyrin II. The uroporphyrin formed was found to be a mixture of uroporphyrin, "pseudo-uroporphyrin", and smaller amounts of intermediate penta- or hexa-carboxylic porphyrin. It is presumed that uroporphyrin II is formed, although this is indistinguishable from uroporphyrin I in the chromatographic procedure of Falk and Benson[12]. These results suggest that crude enzyme preparations are capable of splitting off an aminomethyl group from the $\alpha\alpha'$-substituted dipyrromethane (IV).

TABLE I

EFFECT OF CRUDE ENZYME PREPARATION ON DIPYRROMETHANES

Acetone-dried *R. spheroides* (20 mg) incubated for 4 h semi-anaerobically at 37° in citrate–phosphate buffer, pH 6.5, with dipyrromethanes (100 μg) in a total vol. of 0.5 ml. Controls containing all components except acetone-dried cells were incubated under the same conditions. 1 drop each of aq. I_2 (1 % w/v) and $Na_2S_2O_3$ (1 % w/v) were added at the end of the incubation period; porphyrins were extracted and estimated as previously described[10].

Substrate		*Porphyrin formed (μg)*		
		Uro-	*Copro-*	*Total*
Compounds (I), (II) and (III)	Controls	Nil	Nil	Nil
	Tests	Nil	Nil	Nil
Compound (IV)	Controls	5	Nil	5
	Tests	16	29	45

Formation of coproporphyrin II is probably due to the non-specific uroporphyrinogen decarboxylase present in the crude preparations[5, 13].

None of the above four dipyrromethanes was converted to porphyrins by a partially purified preparation of PBG-deaminase. The dipyrromethanes were also tested as possible inhibitors of PBG-deaminase. Compound (IV) was not inhibitory at the concentration tested (10^{-3} *M*). The dipyrromethanes (I), (II), and (III) were, however, inhibitory although the inhibition was non-competitive (Fig. 1). This contrasts with the observation that the corresponding *αα'*-unsubstituted monopyrrole, opso-pyrrole carboxylic acid, is a competitive inhibitor of PBG-deaminase[14, 15].

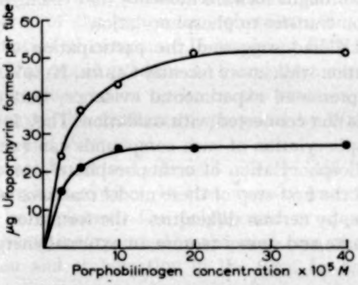


Fig. 1. Purified PBG-deaminase (1170 μg protein) incubated for 30 min in citrate–phosphate buffer, pH 7.6, and different concentrations of PBG with (●) and without (○) dipyrromethane (I) ($2 \cdot 10^{-4}$ *M*) in a total vol. of 20 ml. Uroporphyrin was estimated in the reaction mixture directly after the addition of aq. I_2 (1 %, w/v) and $Na_2S_2O_3$ (1 %, w/v) to oxidise uroporphyrinogen to uroporphyrin, followed by acidification to 5 % (w/v) HCl[5]. Dipyrromethane solutions became pink on standing in acid solution; the pink pigment was non-fluorescent and may be due to polypyranes; this did not interfere with the uroporphyrin estimations under the above conditions.

Department of Chemical Pathology,
University College Hospital Medical School,
London (Great Britain)

D. S. Hoare*
H. Heath

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* Present address: A.R.C. Unit for Microbiology, University of Sheffield, Yorkshire (Great Britain).

Preliminary Notes

Oxidative and photosynthetic phosphorylation involving 2-methylquinones

Recently HATEFI[1] has brought forward evidence that coenzyme Q (ubiquinone) is a factor coupling electron transfer to phosphorylation.

In 1954, WESSELS[2] had suggested the participation of quinol phosphates in oxidative phosphorylation while more recently CLARK, KIRBY AND TODD[3], HARRISON[4] and WIELAND[5] have presented experimental evidence, that quinol phosphates can undergo dephosphorylation connected with oxidation. They found in model reactions that oxidative dephosphorylation of such compounds can result in the formation of pyrophosphate (*i.e.* phosphorylation of orthophosphate[3]), or of ethanol phosphate[5].

The occurrence of the first step of these model reactions in the natural systems is accompanied, however, by certain difficulties—the formation of a quinol phosphate from inorganic phosphate and quinol require an external energy supply.

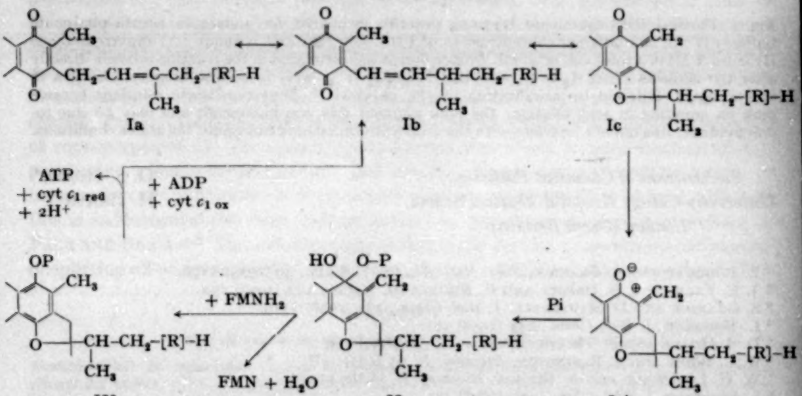
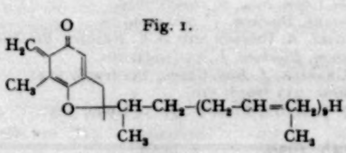


Fig. 1.



IV

Abbreviations: FMN, FMNH$_2$, oxidized and reduced flavine mononucleotide; ADP, ATP, adenosine di- and triphosphate; Cyt., cytochrome; P$_i$, inorganic phosphate.

This difficulty may be omitted by accepting the modified form of natural 2-methyl-3-polyprenoid quinones, corresponding to the active form of vitamin K, proposed in 1950[6,7].

The proposed mechanism for the participation of such an active form in electron transfer and ATP synthesis is presented in Fig. 1.

The structure of 2-methyl-3-polyprenoid quinones may be expressed by the isomeric para-quinonoid formulae Ia and Ib, and the ortho-quinonoid Ic, the latter corresponding to the active form of vitamin K, previously suggested[6,7]. In Ic, a charge separation may occur in the C=O group of the orthoquinonoid system $O{=}\overset{|}{C}{-}\overset{|}{C}{=}CH_2$ (stabilized by a heterocyclic ring), forming Ic′ without an external energy supply. This opens the possibility of the proton and phosphate-ion addition to form the compound II. The reduction of compound II involving $FMNH_2$, would lead to the quinol phosphate III. This would produce ATP and regenerate the initial quinone, thus completing the phosphorylation cycle, in the reaction with ADP and ferricytochrome c_1, according to the mechanism, proposed by WESSELS[2], CLARK, KIRBY AND TODD[3], HARRISON[4], and WIELAND[5].

If structure Ic cannot be formed, the molecules Ia and Ib may act as electron carriers, but not as phosphate carriers. In that way the uncoupling of oxidation and phosphorylation may be explained. The polyprenoid or appropriate side chain in position 3, capable of being incorporated in the lipid core of the lipoprotein, would condition the formation and stabilization of the form Ic, and thus the oxidative phosphorylation.

A similar mechanism may be suggested for photosynthetic phosphorylation involving the active form of the plant quinone Q_{254}, expressed by formula IV.

Department of Biochemistry, Warsaw University (Poland) IRENA CHMIELEWSKA

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Insulin-mediated effect on the activity of UDPG-glycogen transglucosylase of muscle

The increased accumulation of glycogen found in the presence of insulin in previous experiments appeared to be difficult to explain solely on the basis of increased glucose uptake[1], and suggested the possibility of an insulin-mediated effect on the enzymes of the glycogen cycle. We have therefore measured the activities of phosphoglucomutase, UDPG pyrophosphorylase, UDPG-glycogen transglucosylase, and phosphory-

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Abbreviations: UDPG, uridine diphosphate glucose; G-6-P, glucose 6-phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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lase in parallel extracts of control and insulin-treated diaphragms. An increased activity of UDPG-glycogen transglucosylase has been found in extracts of diaphragms incubated with insulin as compared to controls. As this enzymic activity appears to be rate-limiting in glycogen synthesis[2], an increased efficiency in this step could explain the accumulation of glycogen observed.

Isolated cut rat diaphragms were used, employing the techniques of incubation, freezing, and extraction of the tissues previously described[1]. The powdered frozen diaphragms were homogenized either in 0.1 *M* NaF – 0.002 *M* EDTA[3], for the measurement of phosphorylase activity, or in 0.05 *M* Tris-maleate – 0.0025 *M* EDTA (pH 8.2), for phosphoglucomutase, UDPG pyrophosphorylase, and UDPG-glycogen transglucosylase activities. Extracts were prepared by centrifugation of the homogenates at 20,000 × *g* for 30 min*.

Phosphorylase activity was measured at pH 6.1 by the method of ILLINGWORTH AND CORI[4], with the results expressed in µmoles/g/h. Phosphoglucomutase and UDPG pyrophosphorylase activities were determined spectrophotometrically[5]. Table I shows that no significant difference was observed in any of these three activities.

TABLE I

PHOSPHOGLUCOMUTASE, UDPG PYROPHOSPHORYLASE AND PHOSPHORYLASE ACTIVITIES OF EXTRACTS OF CONTROL AND INSULIN-TREATED DIAPHRAGMS

Mean values (µmoles/g tissue/h) of 6 experiments ± standard error of mean.

	Control	*Insulin*	*Mean difference*	*P*
Phosphoglucomutase	7484	7671	187 ± 100	> 0.15
UDPG pyrophosphorylase	440	437	3 ± 15.6	> 0.5
Phosphorylase, total§	2262	2364	102 ± 148	> 0.5

§ The ratios of a/total phosphorylase activities found in these experiments (control, 87; insulin 85, $P > 0.3$) appear to be much higher than previously reported values. This discrepancy may be due to the increase in the proportion of phosphorylase *a*[6] that occurs when freezing is used as a method of fixation.

TABLE II

UDPG-GLYCOGEN TRANSGLUCOSYLASE ACTIVITY

Mean results (µmoles/g tissue/h) of 13 experiments.

	Control	*Insulin*	*Mean difference*	*P*
No G-6-P in test	13.2	18.0	4.8 ± 0.5	< 0.001
G-6-P added	59.8	58.4	1.4 ± 3.4	> 0.50

For the determination of UDPG-glycogen transglucosylase activity, [^{14}C-*glucose*]-UDPG and glycogen were incubated at pH 8.2 with the tissue extracts, with or without added G-6-P. After 10–20 min at 30° ,the incubation mixtures were deproteinized by addition of 5 % trichloroacetic acid, glycogen was isolated from aliquots of the supernatant, and its radioactivity measured. In Table II are presented the results of these determinations.

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* No loss of UDPG-glycogen transglucosylase activity was found in the extracts, as compared with homogenates.

It has been found previously[1] that the G-6-P content is increased in insulin-treated as compared to control diaphragms. To test the possibility that the observed difference in activity of UDPG-glycogen transglucosylase could be accounted for by an increase in a free or loosely bound co-factor, the enzyme was precipitated by $(NH_4)_2SO_4$ (60 % satn.), the precipitates washed with 60 % satd. $(NH_4)_2SO_4$, and dialyzed (1–3 h). After this treatment, the increased activity of insulin extracts persisted when tested in the absence of added G-6-P. Recovery of enzyme activity was virtually complete. Furthermore, when extracts were incubated at [illegible]0° with a large excess of added G-6-P prior to the $(NH_4)_2SO_4$ treatment, the activity recovered (measured in the absence of added G-6-P in the test system) accounted for only that initially present, which appears to indicate that added G-6-P is not tightly bound to the enzyme.

It is known[1] that high glucose concentrations in the incubation medium may increase the G-6-P content of diaphragms even over that found in diaphragms incubated with insulin at lower glucose concentrations; however, the glycogen content is not correspondingly increased. Accordingly, diaphragms were incubated at medium glucose concentrations between 70 and 360 mg/100 ml without added insulin. No differences were observed at different concentrations in the medium (6 experiments) in the UDPG-glycogen transglucosylase activity of diaphragms incubated without added insulin in the face of a consistently higher activity of extracts prepared from insulin-treated diaphragms.

From these results it appears that observed differences in UDPG-glycogen transglucosylase activity cannot be explained on the basis of an increased G-6-P content of diaphragms incubated with insulin. The activity of extracts measured in the presence of G-6-P seems to remain constant whatever the activity determined in the absence of added G-6-P may be: a reasonable explanation would be the existence in muscle of two species of UDPG-glycogen transglucosylase: one active in the absence of added G-6-P, the other requiring it for activity. The insulin effect here reported could then be interpreted as an interconversion between the two species.

It has been demonstrated that in the presence of insulin increased glycogen synthesis occurs associated with an increased activity of UDPG-glycogen transglucosylase. This finding represents additional evidence for assigning the synthetic role in the glycogen cycle to the uridine-linked enzymes.

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Department of Pharmacology,
School of Medicine, Western Reserve University,
Cleveland, Ohio (U.S.A.)

CARLOS VILLAR-PALASÍ
JOSEPH LARNER

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Dissociation constants of peptides

V. Continuous infrared spectroscopy in aqueous solutions between 1,000 and 5,000 cm^{-1} (2 to 10 μ)

While investigating the relation between dissociation constants of peptides and configuration in aqueous solution[1], a technique was developed for the determination of continuous infrared spectra in aqueous solutions between 1,000 and 5,000 cm^{-1}. Hitherto, such measurements were limited either to the range 1,600–1,000 cm^{-1} (6–10 μ) for H_2O solutions[2–5], or they required determinations both in H_2O and D_2O, in order to penetrate the intense H_2O bands near 3,300 and 1,600 cm^{-1} (3 and 6 μ)[6,7].

Measurements were carried out in a Beckman IR-4 spectrophotometer, using CaF_2 cells. Spacers were cut from 0.001 inch (0.025 mm) Mylar film (DuPont Co., Wilmington, Del.), which permitted acceptable matching by varying the pressure on the clamping screws of the cells. Closeness of match was checked before each experiment with solvent in both cells between 1,500–1,700 and 3,000–3,630 cm^{-1}, where H_2O absorbs strongest. Instrument response was so adjusted as to present a correct spectrum of polystyrene, when a film of this material was superimposed on the cell in the sample beam.

In Fig. 1–3 are shown typical spectra which illustrate the effects of solute concentration, pH and solute "type". The small curves on the right-hand edges in Fig. 1 represent controls with solvent in both cells. The dotted lines in some spectra near 1,650 and 3,500 cm^{-1} represent the phenomenon of "negative absorption". In these cases, the cell containing the solute was more transparent than that containing the solvent, and the dotted curves were obtained when the cells were interchanged in the beams.

From thermodynamic measurements[8–10] it had been inferred that glycine and

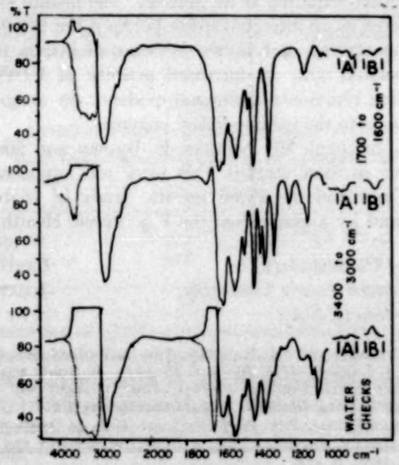

Fig. 1. From top to bottom: infrared spectra of 1 *M* solutions of glycine, L-alanine and L serine in H_2O.

serine behave in water as strong "structure breakers", and alanine less so. This may account for the strong "negative absorption" bands shown in Fig. 1, since disruption of the hydrogen-bonded OH frequencies of water should make the solution cell less opaque than the solvent cell. That this phenomenon is at best only partly due to the difference in the concentration of the water in the two cells is shown in Fig. 2, where the negative absorption seems to be associated with the $-NH_3^+$ group of the solute. It is not observed in alanylalanine (LL), Fig. 3, but a small amount of negative absorption remains in alanylalanine (LD) even at pH 6.5. This may support a deduction previously made[11], when the first ionization of alanylalanine (LL) was closely linked with increased water structure, whereas that of its (LD) isomer was considered not to involve the solvent at all. The entropies of ionization[1,11] are consistent with a mechanism involving "structure breaking" in the (LD) peptide, but require some increased water structure in the (LL) peptide. On the basis of such a hypothesis, negative absorption would be expected with the (LD), but not with the (LL) peptide.

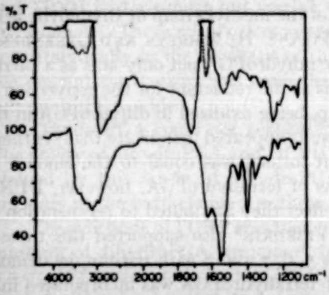

Fig. 2. Infrared spectra of 0.5 M solutions of L-alanine at pH 0.5 (top) and pt 11.5 (bottom).

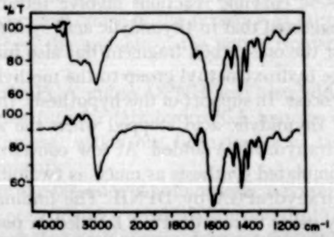

Fig. 3. Infrared spectra of 0.3 M solutions of H·Ala·Ala·OH (LD), top, and H·Ala·Ala·OH (LL), both at pH 6.5.

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Department of Biochemistry and Nutrition, ERIC ELLENBOGEN*
Graduate School of Public Health, University of Pittsburgh,
Pittsburgh 13, Pa. (U.S.A.)

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* U.S. Public Health Service Special Research Fellow 1959–60 at Dept. of Biophysics, Weizmann Institute of Science, Rehovoth, Israel. Presented in part at the 135th National Meeting, Am. Chem. Soc., *Abstracts*, p. 47C, 1959.

The role of DPNH and tetrahydropteroylglutamate in the synthesis of thymidylic acid

Extracts of bacteria and mammalian thymus gland can utilize formaldehyde or the hydroxymethyl group of serine as precursors of the methyl group of thymidylic acid. These enzymic reactions involve tetrahydroPGA[1,2]. HUMPHREYS AND GREENBERG[2] suggested that in thymidylic acid synthesis tetrahydroPGA not only acts as a carrier for the one-carbon fragment but also functions as the reductant for the conversion of the hydroxymethyl group to the methyl group, being oxidized to dihydroPGA in the process. In support of this hypothesis, their results appeared to indicate that synthesis of thymidylic acid stopped when the amount formed was equal to the amount of tetrahydroPGA added. At low concentrations of tetrahydroPGA, however, DPNH stimulated synthesis as much as twofold, an effect they attributed to regeneration of tetrahydroPGA by DPNH. The findings of FRIEDKIN[3] also supported this theory. By using tetrahydroPGA labelled in positions 5, 6, 7 and 8 with tritium, he demonstrated that hydrogen from these positions of tetrahydroPGA was incorporated into thymidylic acid. There was a 5–6 fold dilution of the specific activity of the hydrogen incorporated, however, a result which is difficult to explain by isotope selection.

An alternative hypothesis is as follows. The reduction of TPN by tetrahydroPGA in the presence of dihydroPGA reductase has been reported by OSBORN AND HUENNEKENS[4]. Moreover, the reductase also functions with DPN[5]. In experiments where tetrahydroPGA is the only added reducing agent, it is possible that tetrahydroPGA acts by keeping in the reduced state catalytic amounts of DPN or TPN which are strongly bound to protein. This bound DPNH or TPNH may then act as the actual reductant in the methylation reaction.

From the available evidence it is not clear which of these two hypotheses represents the actual sequence of reactions. It was therefore desirable to obtain more experimental information about the role of DPNH and this has been achieved by a study of the effect of aminopterin on the system. The results here presented indicate that DPNH stimulates thymidylic acid synthesis by regenerating tetrahydroPGA rather than by acting as reductant in the methylation reaction.

Sonic extracts of *Streptococcus faecalis* R, after treatment with streptomycin sulphate and Dowex-1-chloride, synthesized [*Me*-^{14}C]thymidylic acid from uracil deoxyriboside-5′-phosphate and [3-^{14}C]serine. No thymidine was formed during the reaction and uracil deoxyriboside was not utilised for synthesis of thymidylic acid or thymidine. All experiments were performed in the presence of 2,3-dimercaptopropanol which is necessary to protect tetrahydroPGA and dihydroPGA from oxidative decomposition. TetrahydroPGA was essential for thymidylic acid synthesis. In the presence of 5 μmoles *dl*-tetrahydroPGA, prepared by catalytic hydrogenation, neither DPNH nor TPNH stimulated synthesis of thymidylic acid. Since *dl*-tetrahydroPGA was used in these experiments and, as only one isomer is reactive enzymically, the effective concentration was half that added. 50 mμmoles of the active isomer of tetrahydroPGA permitted the synthesis of 45 mμmoles of thymidylic acid in 6 h*, so that tetrahydroPGA here acted as substrate rather than as a catalytic coenzyme. The further addition of glutathione to the system had no effect. However, when 2 μmoles DPNH were added, thymidylic acid synthesis increased to 302 mμmoles and TPNH had a similar but smaller effect, stimulating synthesis to approximately 150 mμmoles. These results demonstrate for the first time that tetrahydroPGA can act catalytically in the synthesis of thymidylic acid, since in the presence of DPNH the amount synthesized was more than 6 times the amount of the active isomer of tetrahydroPGA added.

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* The reaction mixture contained, in 1 ml, 5 μmoles uracil deoxyriboside-5′-phosphate, 0.5 μmole pyridoxal phosphate, 2.5 μmoles 2,3-dimercaptopropanol, 5.35 μmoles [3-^{14}C]L-serine ($4.96 \cdot 10^5$ counts/min), 100–200 units serine transhydroxymethylase[6], buffered with 0.1 *M* N-ethyl morpholine, pH 7.8. The reaction was carried out under N_2. Thymidylic acid was isolated from the mixture (after addition of carrier) by paper chromatography and degraded to iodoform[7]. The purified iodoform was dissolved in toluene and counted in a Tricarb liquid scintillation counter.

DihydroPGA could not replace tetrahydroPGA unless DPNH was also added. The ability of dihydroPGA and DPNH to substitute for tetrahydroPGA may be explained by the action of dihydroPGA reductase, which has been found in large amounts in the extracts used in these experiments.

The synthesis of TDRP was then tested in the presence of aminopterin, a powerful inhibitor of dihydroPGA reductase[8]. In the presence of 5 μmoles *dl*-tetrahydroPGA, $7.2 \cdot 10^{-5}$ *M* aminopterin had no effect, showing that aminopterin does not inhibit the methylation reaction. Similarly, when 100 mμmoles *dl*-tetrahydroPGA were used, aminopterin ($5 \cdot 10^{-5}$ *M*) did not diminish thymidylic acid synthesis. This demonstrated that since aminopterin does not inhibit the methylation process, dihydrofolic reductase cannot be an essential participant in that process, *i.e.* the second of the hypotheses discussed above is not correct.

When aminopterin was added to the system in which tetrahydroPGA was acting catalytically in thymidylic acid synthesis, however, $5 \cdot 10^{-5}$ *M* aminopterin entirely inhibited the additional synthesis resulting from the addition of 2 μmoles DPNH. Furthermore, synthesis was completely inhibited by aminopterin when tetrahydroPGA was replaced by dihydroPGA and DPNH. In these experiments, therefore, the effect of DPNH is mediated entirely by dihydrofolic reductase by which the DPNH regenerates tetrahydroPGA. This supports the hypothesis that tetrahydroPGA is oxidised to dihydroPGA during thymidylic acid synthesis and tetrahydroPGA may be regenerated by a reductant such as DPNH.

Department of Biochemistry,
John Curtin School of Medical Research,
Australian National University,
Canberra (Australia)

BARBARA M. MCDOUGALL
R. L. BLAKLEY

[1] M. FRIEDKIN AND A. KORNBERG, in W. D. MCELROY AND B. GLASS, *The Chemical Basis of Heredity*, The John Hopkins Press, Baltimore, 1957, p. 609.
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Abbreviations: tetrahydroPGA, 5,6,7,8-tetrahydropteroglutamic acid; dihydroPGA, dihydropteroylglutamic acid; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; TDRP, thymidylic acid.

On the mechanism of a photodynamic reaction

During work on the mechanism of action of the fungicide pimaricin on yeast cells it was observed that visible light destroys the pimaricin in an aqueous solution if riboflavin is present[1]. This effect is remarkable for pimaricin does not show any absorption in the visible region. Since the presence of riboflavin is obligatory for the destruction of the pimaricin, it appears that the riboflavin absorbs the light and brings about the destruction of pimaricin. Pimaricin belongs to a group of polyene antibiotics[2-4]. Though the structure of the molecule is rather complicated, the part of the molecule which is responsible for the ultraviolet spectrum is practically limited to a tetraën grouping. Compounds with a reactive system of conjugated double bonds are usually very sensitive to free radicals and so we originally supposed the photodynamic destruction to be due to free radicals, which could have their origin in the decomposition of riboflavin by light[1].

Though the molecule of pimaricin is very complex we have continued to use this compound since a change under the influence of light can be easily detected spectrophotometrically. It would seem that a more simple tetraën might be better suited but such compounds are rare, are generally very labile and usually too insoluble in water. Pimaricin, on the other hand, has several advantages; it is commercially available*, it is relatively stable and the solubility is sufficient for the experimental conditions.

Pimaricin has maxima at 281, 292, 304.5 and 319 mμ. The spectrum of riboflavin is more complicated with maxima at 223, 268, 373 and 445 mμ (Fig. 1).

Several photodynamic destructions in which riboflavin is involved have been described in the literature, *e.g.* aerobic photooxidation of phenols, ascorbic acid,

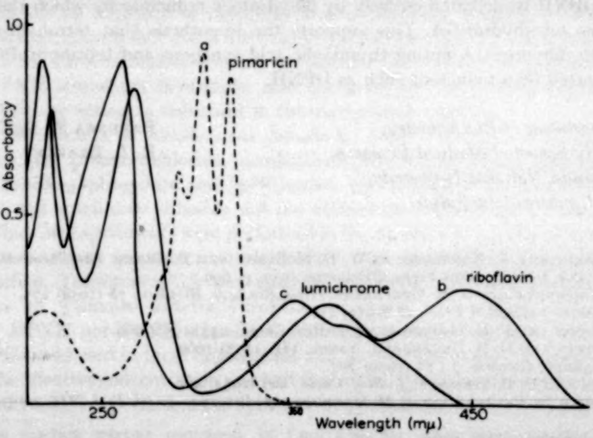

Fig. 1. Absorption spectrum of pimaricin, riboflavin and lumichrome in water. (a), Pimaricin ($1.44 \cdot 10^{-5}$ *M*); (b), riboflavin ($2.66 \cdot 10^{-5}$ *M*); (c), lumichrome ($2.24 \cdot 10^{-5}$ *M*). Light path, 1 cm.

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* We wish to express our gratitude to the Royal Dutch Fermentation Industries, Delft, Holland, for supplying this fungicide.

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3-indoleacetic acid and histidine[5,6]; usually an oxidation of the substrate or a free-radical mechanism is supposed to occur[7-10]. From experiments to be described it will be clear that neither is acceptable as an explanation for our observations of the photodynamic behaviour of the system riboflavin–pimaricin.

As source of irradiation we used a Kromayer high-pressure mercury lamp 3 cm from the aqueous solution. With an interference filter, light with a wavelength of about 443 mμ was obtained. The destruction of pimaricin could be followed by measuring the absorbancies at 304 and 319 mμ by means of an Unicam Spectrophotometer SP 500. 23 % of the pimaricin was destroyed after 5 min of irradiation. The absorption spectrum of riboflavin remained unchanged under these conditions and no pimaricin destruction could be observed by irradiating pimaricin alone.

Though riboflavin decomposes by prolonged irradiation, yielding lumichrome, a free-radical mechanism is not likely, because lumichrome alone is also able to bring about this pimaricin destruction. Lumichrome, however, is not decomposed by the light used in these experiments (368 mμ and 395 mμ) and even not by u.v. light of shorter wavelengths*. Moreover the photolysis of riboflavin in alcohol is much stronger than in water and nevertheless no destruction of pimaricin takes place in alcohol.

Vernon[9,10] has suggested a free-radical mechanism in which excited riboflavin decomposes water and the OH radicals thus formed would oxidize the substrate, or form H_2O_2 in absence of a substrate. We have found, however, that no H_2O_2 was formed in irradiated aqueous solutions of lumichrome. Lumichrome appears to be completely stable to ultraviolet radiation*, but addition of a very small amount of H_2O_2 ($< 10^{-6}\,M$) brings about a very rapid destruction of lumichrome and so any H_2O_2 formed would be easily detected. If no H_2O_2 arises under the experimental conditions the formation of OH radicals is also not very probable.

If oxidation of pimaricin, accompanied by the simultaneous reduction of riboflavin, were the primary cause of the reaction between riboflavin and pimaricin it might be expected that oxygen would accelerate the photodynamic reaction. However, the destruction of pimaricin appeared to be less under these conditions, while bubbling through N_2, prior to illumination, strongly accelerated the pimaricin destruction.

The remarkable inhibiting effect of oxygen on the photodynamic destruction of the systems riboflavin–pimaricin and lumichrome–pimaricin suggests a direct transfer of the excitation energy from riboflavin or lumichrome to pimaricin without free radicals being involved. Arguments will be given in a future paper that a triplet–triplet transfer is the most acceptable explanation of the phenomenona described.

Biochemical Laboratory of the Technological University,
Julianalaan 67, Delft (The Netherlands)

E. Zondag
J. Posthuma
W. Berends

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The use of action spectra to determine the physical state of DNA *in vivo*

The purpose of this note is to point out a way to use action spectra to distinguish between biological actions which are carried out by single- or double-stranded polynucleotides. The proposed experimental technique has theoretical and experimental foundations. The theoretical basis is that the pyrimidines are more sensitive than the purines to ultraviolet light[1–3]. A precise prediction of the relative sensitivities of cytosine and thymine in a biological-inactivation process is difficult because there are rapid reversible reactions which take place in the irradiation of the free bases. The speed of the reverse reaction is much greater for thymidine than for cytosine[4]. If we ignore this complication, we would predict that the efficiency of different wavelengths of ultraviolet light in destroying the biological function of a single-stranded DNA chain (the action spectrum), should be similar to the absorption spectrum of a sum of cytosine plus thymine (which has a minimum near 2400 Å), rather than like the absorption spectrum of DNA which has a minimum near 2300 Å[5].

In a double helix the purines and pyrimidines are tightly coupled to one another in a regular way. An absorbed quantum may be thought of as transferred from one base to another[6]. A quantum absorbed in a purine may be transferred to a pyrimidine which is then affected. Thus for double helices all absorbed quanta should have approximately equal efficiencies for biological inactivation and the action spectrum should be similar to the absorption spectrum of DNA.

The prediction of differences between the action spectra of single and double strands of DNA has some theoretical uncertainties and objections. The data on the sensitivity of the bases to ultraviolet light have been obtained at 2537 Å, and we have assumed that the effects are wavelength-independent. Wavelengths below 2300 Å produce different effects than the reversible ones found at 2537 Å[7]. The contribution to the biological sensitivity which may arise from quanta absorbed in nearby protein components has been deliberately ignored because much larger energies are needed to affect proteins than nucleic acids[8] and because there has been no demonstrated energy transfer from protein to nucleic acids in systems such as tobacco mosaic virus[9].

The experimental data which indicate that the affected bases in two-stranded DNA are the pyrimidines are those of Errera[10], who showed that irradiation of DNA destroyed the pyrimidines before the purines, and of Shugar[11], who has shown that the cross-linking of DNA involves mainly the pyrimidines. The action spectrum[12] for the gelling, or cross-linking, of dry thymus DNA is shown in Fig. 1. It is similar to the absorption spectrum of DNA.

There exist only a few action spectra on well-defined polynucleotides which may be used to investigate the differences between single- and double-stranded structures. Three examples that are simple (but are still not as simple or as accurate as one would desire) are those of ΦX174, a single-stranded DNA virus[13], T2 bacteriophage, a primarily double-stranded DNA virus[14], and the gelling of thymus DNA. The action spectrum for the inactivation of ΦX174, as determined by the author, is shown in Fig. 1. It is to be compared with the spectrum obtained by ZELLE AND HOLLAENDER[15] for T2. The double-stranded DNA structures have minima at 2300 Å and the single-stranded one has a minimum at 2400 Å. Further evidence for the important role played by light absorption in the pyrimidines in the inactivation of single-stranded DNA comes from the change in the action spectrum of ΦX174 when the irradiation is performed at pH 2 (ref. 16). The change is similar to the change in cytosine absorption. These data indicate that light absorption in the protein part of the virus is unimportant in inactivation because the protein spectrum does not change in this way with pH.

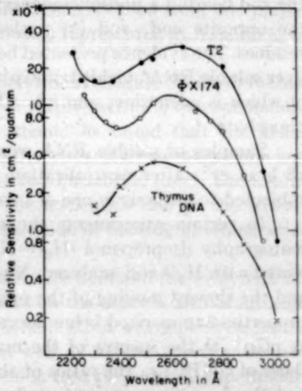

Fig. 1. The action spectra for the inactivation of T2 bacteriophage[15], the gelling of thymus DNA[12], and the inactivation of the virus ΦX174. The first two spectra are of effects on double helix DNA and show minima at 2300 Å. ΦX174, a single strand DNA virus, has a minimum at 2400 Å. The data for T2 are those of ZELLE AND HOLLAENDER [15].

The proposed method for distinguishing between single- and double-stranded DNA *in vivo* involves a reinterpretation of action spectra usually described as "nucleoprotein". The rise in sensitivity at low wavelengths probably indicates an effect on cytosine rather than on protein. The differences between the two types of DNA action spectra are small but they are larger than the hyperchromic effect used to distinguish single from double strands *in vitro*.

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Biophysics Department, Yale University,
New Haven, Conn. (U.S.A.)
RICHARD SETLOW

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Studies on soluble ribonucleic acid of rabbit liver. Terminal groups and nucleotide composition

It is well established[1] that alkaline hydrolysis of a polyribonucleotide of the general structure pNpNpN . . . . pNpN (where N represents purine or pyrimidine ribonucleosides) will yield (1) a mixture of nucleoside 2′,5′- and 3′,5′-diphosphates from the end bearing a phosphomonoester group at a C-5′ hydroxyl; (2) nucleosides from the opposite end; and (3) nucleoside 2′- and 3′-monophosphates from all other residues. The evidence presented below indicates that upon alkaline hydrolysis rabbit-liver soluble RNA[*] yields (1) pGp; (2) a mixture of nucleosides, the major component of which is adenosine; and (3), AMP, UMP, CMP, GMP, and 5-ribosyluracil monophosphate[2,3].

Samples of soluble RNA were hydrolyzed in the presence of 0.3 *N* KOH for 18 h at 37°; after neutralization with Dowex-50, H^+ form, the hydrolysates were subjected to analysis in one of several ways.

In certain experiments the sample was subjected to descending paper chromatography (isopropanol-H_2O-NH_3)[4]. The bands seen under ultraviolet light were eluted with H_2O and analyzed. Nucleoside diphosphates are found between the origin and the slowest moving of the nucleoside monophosphates, GMP ($R_F = 0.25$)[5]. The properties summarized below served to identify the material in the diphosphate area as pGp: (1) the spectra of the material at pH 2 and 12 are almost identical with those of GMP[6], *i.e.*, the ratios of absorbancy at 250 mμ/260 mμ and 280 mμ/260 mμ, and the λ_{max} are 0.90, 0.66, 258 mμ, and 0.91, 0.61, 258 mμ, at pH 2 and 12, respectively; (2) $\varepsilon_{(P)}$ at 258 mμ at pH 2 is 6.5; (3) the electrophoretic mobility at pH 7 is 1.4 times that of GMP[7]; (4) the material does not react with periodate[8]; (5) hydrolysis in 1 *N* HCl for 1 h at 100° gives a single product which has the chromatographic and spectral properties of guanine[6].

In another procedure the neutralized hydrolysate was subjected to paper electrophoresis at pH 7. Under these conditions all nucleoside diphosphates migrate ahead of the monophosphates[7]. The ultraviolet-absorbing area ahead of the monophosphates was eluted and its spectrum at pH 2, 7, and 12 confirmed the observation that pGp represents at least 80 % of the nucleoside diphosphates.

The two major bands on the isopropanol-H_2O-NH_3 chromatograms were analyzed by chromatography on Dowex-1, formate form[9]; these fractions contained the nucleoside monophosphates in the following molar ratios: AMP, 1.0; UMP, 1.0; GMP, 1.8; CMP, 1.7; and 5-ribosyluracil monophosphate, 0.2. The high proportion of 5-ribosyluracil in soluble RNA has also been reported by other workers[10–12].

In an alternative analytical procedure the neutralized hydrolysate was placed on a column of diethylaminoethylcellulose and the column eluted with an $(NH_4)_2CO_3$ gradient at pH 8.7. The components of the hydrolysate are eluted in the following sequence: nucleosides, CMP, AMP, UMP, 5-ribosyluracil monophosphate, 2′-GMP

Abbreviations: RNA, ribonucleic acid; AMP, UMP, GMP, CMP, the mixed 2′- and 3′-isomers of adenylic, uridylic, guanylic and cytidylic acids, respectively; pGp, mixed guanosine-2′,5′-, and -3′,5′-diphosphate; polynucleotides are written so that a small p to the left of a nucleoside residue indicates esterification at a C-5′ hydroxyl and a small p to the right of a nucleoside residue esterification at a C-3′ hydroxyl.

[*] Rabbit-liver soluble RNA was isolated from the soluble portion of rabbit-liver homogenates by a NaCl-extraction procedure that will be fully described in a future publication. The preparation serves as an acceptor for activated amino acids.

and 3'-GMP. The molar ratios of the monophosphates were the same as those stated above.

Examination of the nucleoside fractions of the diethylaminoethylcellulose columns and the isopropanol–H_2O–NH_3 chromatograms (fastest-moving material) revealed the presence of adenosine (identified by spectra, paper chromatography, and adenosine deaminase), cytidine (identified by spectra), and smaller amounts of other nucleosides. The relative amounts of the various nucleosides varied from one preparation of soluble RNA to another. In general, adenosine represented at least 50 % of the total nucleoside.

The data summarized above suggest that the polyribonucleotide chains of rabbit-liver soluble RNA have the structure pGpNpN . . . . pNpN, with a 5'-phosphomonoester group on a terminal guanosine residue. It should be noted that the molar quantities of pGp and nucleosides isolated were of the same order of magnitude and both were consistent with a chain length of about 100 nucleotide units. The identification of adenosine and cytidine, arising from the nucleoside end of the soluble RNA chain, is consistent with recent reports on the mode of binding of amino acids to soluble RNA[13–15].

The near equivalence between the content of adenylic acid and the uridylic acids on the one hand, and cytidylic and guanylic residues on the other is also of interest. The implications of these data for the structure of soluble RNA will depend on results obtained with soluble RNA from other species as well as further investigations of secondary structure[16]. It is of interest in this connection that preliminary experiments in this laboratory have shown that rabbit-liver soluble RNA is capable of reacting with formaldehyde[17].

Laboratory of Biochemistry and Metabolism, MAXINE F. SINGER
National Institute of Arthritis and Metabolic Diseases,
Laboratory of Cellular Pharmacology, GIULIO L. CANTONI
National Institute of Mental Health, National Institutes of Health,
Bethesda 14, Md. (U.S.A.)

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Lack of mandatory coupling between RNA and protein synthesis

That RNA plays an important role in protein synthesis has been amply demonstrated. However, the specific mechanisms by which RNA mediates protein synthesis still present unsolved problems and controversial areas. One such controversy revolves around the question whether new RNA synthesis is mandatorily coupled with new protein synthesis. Evidence is presented herein clearly demonstrating that such mandatory coupling does not exist since protein synthesis can be shown to occur in the absence of RNA synthesis.

In these experiments, penicillin-spheroplasts[1] of *Escherichia coli* K12 were incubated in a mixture of 5 amino acids (glutamate, asparate, leucine, isoleucine, valine), β-galactosidase inducer, phosphate buffer and $MgCl_2$. Synthesis of new nucleic acid and protein was determined by precipitation with cold $HClO_4$, and measuring the incorporation of added [1-^{14}C]glycine into the fractions of the washed precipitate which were soluble and insoluble, respectively, in hot trichloroacetic acid. Synthesis of RNA was also determined by measuring the increase in purine ribose by the orcinol method. The increase in purine ribose correlated well with the increase in total nucleic acids as determined by ultraviolet spectrophotometry[2]. Quantitative increases in protein content of the fraction insoluble in hot trichloroacetic acid were measured by the method of LOWRY *et al.*[3] and in some cases by Kjeldahl N determinations. Increases in β-galactosidase were determined in toluene-treated samples by measuring the rate of hydrolysis of *o*-nitrophenyl-β-D-galactoside.

Fig. 1 shows the results of a typical experiment in which incorporation of [^{14}C]-glycine was measured in the presence of two inducers of β-galactosidase synthesis: lactose and TMG. First, it is apparent that when lactose is used as the inducer, the incorporation of ^{14}C into the nucleic acid fraction lags considerably behind incorporation into the protein fraction. Secondly, when TMG is used instead of lactose as the enzyme inducer, protein synthesis, as measured by incorporation of ^{14}C or by synthesis of β-galactosidase, is affected slightly or not at all while RNA synthesis is always inhibited more than 95 %.

The hypothesis that new RNA is required for synthesis of protein is untenable

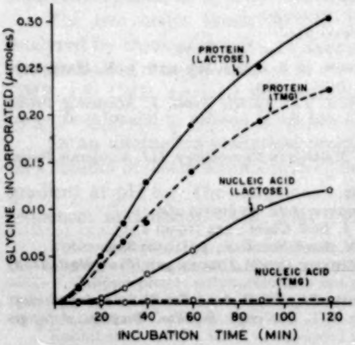

Fig. 1. The effect of inducer on incorporation of [1-^{14}C]glycine into nucleic acid and protein of *E. coli* K12 spheroplasts. 5-ml samples were collected at the indicated intervals, acidified to 5 % $HClO_4$, and washed 3 times with cold 5 % $HClO_4$. The residue was extracted with 4 ml 5 % trichloroacetic acid at 80° for 30 min to separate the nucleic acid from the protein. After washing with 0.25 % trichloroacetic acid, the residue was suspended in 5 *M* NH_4OH for protein determinations. The initial protein content of the 5-ml sample was 2 mg.

Abbreviations: RNA, ribonucleic acid; TMG, methyl-β-D-thiogalactopyranoside.

in view of these results, since protein can be synthesized in the virtual absence of RNA synthesis. The findings by HAHN *et al.*[4] and BEN-ISHAI[5] indicating a requirement for new RNA synthesis were based on concomitant synthesis of RNA and protein after excess RNA was accumulated as a result of prevention of protein synthesis by chloramphenicol[4] or by aureomycin[5]. This conclusion is questionable, however, since RNA formed in the absence of protein would be expected to be non-physiological. With the possible exception of soluble RNA, all RNA functioning in protein synthesis (microsomes, ribosomes) appears to be a component of nucleoprotein particles.

A possible explanation for the results reported in this study is that insufficient energy is available for both protein and nucleic acid synthesis under the conditions described in this work. If protein synthesis occurs preferentially, nucleic acid synthesis would be inhibited when non-metabolizable TMG was used as the inducer, but would not be inhibited in the presence of lactose, which is metabolized after β-galactosidase synthesis. This hypothesis is being presently investigated and a more complete report will be published later.

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General Medical Research Laboratory, VA Hospital;
and Depts. of Biochemistry and Microbiology,
Albany Medical College, Albany, N.Y. (U.S.A.)

RICHARD A. PEABODY
CHARLES HURWITZ

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Role of transfer ribonucleic acid in hemoglobin synthesis

Stimulation of amino acid incorporation into microsomal protein by polynucleotide preparations has been noted by ZAMECNIK *et al.*[1] and WEISS *et al.*[2]. Recently, RENDI AND CAMPBELL[3] have reported increased amino acid incorporation when either soluble or microsomal RNA was added to a preparation containing liver microsomes. In the present studies, incorporation of [^{14}C]amino acid into protein using ribosomes from rabbit reticulocytes was stimulated specifically by transfer RNA[4,5]. The functional integrity of RNA as amino acid acceptor was needed for this stimulation. Previous studies[6,7] have shown that amino acid incorporation in this system represents mainly the synthesis of hemoglobin.

Either transfer RNA[5] from guinea-pig liver or soluble reticulocyte RNA[7] gave comparable increases in [^{14}C]leucine incorporation into protein (Table I) when

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Abbreviations: RNA, ribonucleic acid; CTP, cytidine triphosphate; Tris, tris(hydroxymethyl) aminomethane.

TABLE I

EFFECT OF RNA ON [^{14}C]LEUCINE INCORPORATION

The complete system (minus RNA) contained 2.0 mg enzyme, 7 mg ribosomes from rabbit reticulocytes, 120 µg creatine kinase, 1 µmole ATP, 10 µmoles creatine phosphate, 0.25 µmole guanosine triphosphate, 50 µmoles Tris buffer, pH 7.5, 100 µmoles KCl, 5 µmoles $MgCl_2$, 20 µmoles glutathione, amino acid mixture[6], 0.1 µmole of uniformly-labeled [^{14}C]leucine containing $3 \cdot 10^6$ counts/min/µmole in a final volume of 1.4 ml. The mixture was incubated at 37° for 40 min and then the radioactivity in the total protein determined[6]. The enzyme fraction used was prepared from the soluble fraction of rabbit reticulocytes by protamine treatment and ammonium sulfate fractionation. A more detailed report of the various preparations used will be published elsewhere[7].

RNA added	*Specific activity (counts/min/mg of ribosomes)*
None	643
Transfer RNA*	1591
Reticulocyte soluble RNA	1640
Microsomal RNA	764
Preincubated RNA	684
Preincubated RNA + AS-1 + CTP**	1258

* Transfer RNA (50 µg per assay) was prepared from guinea-pig liver[5], reticulocyte RNA (100 µg) from the total soluble fraction[7]; microsomal RNA (100 µg) from reticulocyte ribosomes by phenol extraction; and preincubated RNA (100 µg) from guinea-pig liver pH 5 enzyme[5,8] by the method of HECHT *et al.*[9].

** AS-1 is an enzyme fraction from guinea-pig liver which contains enzymes which replace terminal groups of preincubated RNA[5]. CTP was used at 0.15 m*M*.

similar levels (assayed by [^{14}C]leucine-RNA formation) were used. Maximal effects were obtained with 25–40 µg of transfer RNA. The increased [^{14}C]leucine incorporation into protein upon addition of RNA in a large number of experiments ranged from 15–20 times the amount of [^{14}C]leucine-RNA which could be formed by this amount of RNA. A "catalytic" role is therefore indicated for RNA in the transfer of amino acid into protein. These results depend on the preparation of soluble enzymes free of transfer RNA.[7] The incorporation shown in the absence of added RNA (Table I) may be due to the presence of a small amount of transfer RNA in the ribosomes. This may also account for the slight stimulation of incorporation by microsomal RNA (Table I). RNA isolated from Turnip yellow mosaic virus or from commercial yeast gave no stimulation of incorporation. These results suggest that amino acid-RNA is an intermediate in hemoglobin synthesis. Other experiments, in which the soluble components were preincubated with [^{14}C]leucine and then excess cold leucine and ribosomes were added and the incubation continued, have indicated that all the [^{14}C]leucine incorporated into protein in this system passes through amino acid-RNA compounds[7].

Preincubated RNA[9], which has lost terminal nucleotides, did not stimulate [^{14}C]leucine incorporation into protein (Table I). This RNA does not form amino acid-RNA unless CTP and an enzyme fraction (AS-1), which replaces terminal nucleotides[5], are added. Similarly, stimulation of incorporation into protein in these studies by preincubated RNA depended on the addition of fraction AS-1 and CTP. Therefore, the stimulation given by RNA is due to its ability to form amino acid-RNA compounds. When AS-1 and CTP were added to the complete system containing transfer RNA, no effect or a slight inhibition was observed. Taken in conjunction with the "catalytic" role shown for RNA, it appears probable that the removal and replacement of terminal nucleotides is not involved directly in hemoglobin synthesis in the cell-free system.

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Biochemistry Department, City of Hope Medical Center,
Medical Research Institute, Duarte, Calif. (U.S.A.)
Esther H. Allen
Richard S. Schweet

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The interrelationships of vitamin B_{12}, β-methylaspartate and thymine

Barker[1] has shown the existence of a coenzyme containing vitamin B_{12}. The coenzyme was demonstrated as a reactant catalyzing the reaction: glutamate $\rightleftharpoons$ β-methylaspartate. One of us[2] has suggested that methylaspartate and its carbamyl derivative are intermediates in thymine biosynthesis and that the vitamin B_{12} requirement for synthesis of deoxynucleic acid could be explained on this basis. Several types of experiments have been conducted to determine possible vitamin B_{12}, β-methyl aspartate, and thymine interrelationships. These were (1) replacement of vitamin B_{12} by β-methylaspartate in the growth of *Poteriochromonas stipitata*, (2) replacement of thymine by β-methylaspartate in the reversal of urethan inhibition of *P. stipitata* and (3) replacement by β-methylaspartate of the thymine requirement of *Escherichia coli* mutants.

The requirement of *Poteriochromonas* for vitamin B_{12} was established by Hutner *et al.*[3] and further studied by Isenberg[4]. In Table I are presented data obtained in growth experiments. Haddow[5] has demonstrated that urethan toxicity to cells can in part be prevented by nucleic acid components, especially thymine. Work in our laboratories[4] has shown that thymine also prevents urethan toxicity to *P. stipitata*. It is shown in Table II that β-methylaspartic acid similarly interferes with urethan inhibition.

The method of Travaglini and Schultz[6] was used to measure the growth response of a thymine-stimulated mutant of *Escherichia coli* ATCC No. 9732h to β-methylaspartate. The response to thymine was minimal but consistent. Data from a typical experiment are shown in Table III.

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TABLE I

β-METHYLASPARTATE AND THE GROWTH OF *P. stipitata*

Inverted-tumbler closed micro-Fernbach flasks containing the following nutrients per 100 ml were inoculated with *P. stipitata*: glucose, 2 g; $CaCl_2$, 0.008 g; $MgSO_4 \cdot 7H_2O$, 0.06 g; KH_2PO_4, 0.1 g; NH_4Cl, 0.05 g; $NaHCO_3$, 0.0169 g; L-isoleucine, L-leucine, L-valine and L-phenylalanine, each 0.05 g; glutamic acid, 0.3 g; L-histidine, 0.1 g; L-arginine, 0.04 g; adenine sulfate, 0.05 g; thiamine, 200 μg; biotin, 2 μg; $ZnSO_4 \cdot 7H_2O$, 0.0352 g; $MnSO_4 \cdot H_2O$, 0.0154 g; $CuSO_4 \cdot 5H_2O$, 0.00197 g; $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 0.002106 g; $CoSO_4 \cdot 7H_2O$, 0.000382 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.00064 g. Where cobalamine was used, it was present at 0.5 μg/100 ml. The response to β-methylaspartate was studied at pH 5.0. After a 7-day incubation at 25° in the light, the 10-ml volume was centrifuged, the cells washed and total cell nitrogen determined.

Medium plus	*Total cell N (μg)*
0	15
Vitamin B_{12}	1688
β-Methylaspartic acid, 0.1 %	715
β-Methylaspartic acid, 0.3 %	613
β-Methylaspartic acid, 0.6 %	326

TABLE II

INHIBITION OF GROWTH OF *P. stipitata* BY URETHAN

The medium used is described in Table I. Vitamin B_{12} was present in all flasks. Total cell nitrogen was determined on washed cells.

Medium plus	*Urethan (%)*	*Total cell N (μg)*
Control	0	3230
	0.2	11
	0.3	11
Thymine, 0.2 %	0.2	2470
	0.3	1680
β-Methylaspartate, 0.2 %	0.2	2560
	0.3	3060
β-Methylaspartate, 0.6 %	0.2	2560
	0.3	3060

TABLE III

STIMULATION OF THYMINE-REQUIRING *Escherichia coli* BY β-METHYLASPARTATE

Basal medium plus	*Growth (+ %)*
No addition	100
Thymine, 0.6 μg/ml	125
Thymine, 1.2 μg/ml	135
β-Methylaspartate, 0.6 μg/ml	167
β-Methylaspartate, 1.2 μg/ml	180
β-Methylaspartate, 2.4 μg/ml	85
β-Methylaspartate, 4.8 μg/ml	60

The data presented in the experiments indicate that methylaspartic acid is stimulatory to organisms requiring either vitamin B_{12} or thymine. None of the other amino acids tried in similar experiments had any stimulating effects. Sufficient work has not been completed to determine if the sparing of thymine in *E. coli* is limited to methylaspartic acid. In the reversal of urethan inhibition thymine and methylaspartate are very active, but glutamate and aspartate have about 20% of the activity of thymine.

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Department of Laboratories, The Long Island Jewish Hospital, New Hyde Park, N.Y. (U.S.A.)

HENRY D. ISENBERG
ELI SEIFTER
JAMES I. BERKMAN

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The characterization of a new metabolite of dopamine

In previous studies we have demonstrated that a neutral metabolite is excreted in rats urine after administration of epinephrine and norepinephrine[1]. AXELROD *et al.*[2] described a compound with similar chromatographic behavior and have identified it as 3-methoxy-4-hydroxyphenylglycol.

In the present communication, we wish to describe the formation, *in vivo*, of a new metabolite of dopamine which has the characteristics of a neutral compound.

A group of rats was injected with 100 μg of a mixture of $[\beta,\beta\text{-}{}^{3}H_{2}]$dopamine and $[\beta\text{-}{}^{14}C]$dopamine with an ${}^{3}H/{}^{14}C$ activity ratio of 1.1/1. Urine was collected over a period of 24 h and hydrolyzed at pH 2. Hydrolyzed urine was extracted 4 times with ethyl acetate. The ethyl acetate fraction was chromatographed in chloroform–acetic acid–water (2:1:1) and then in isopropyl alcohol–aq. ammonia–water (8:1:1). Three radioactive peaks were separated. The peak with the slowest mobility corresponded to authentic 3,4-dihydroxyphenylacetic acid. The mobility of the second peak corresponds to 3-methoxy-4-hydroxyphenylacetic acid, and the fastest-moving peak represents an unknown metabolite. The unknown peak was also chromatographed in butanol–alcohol–water (4:1:1)[3] and its mobility in this solvent system was shown to be different from all other known metabolites of dopamine. This metabolite was demonstrated to be neutral since it was extractable into ethyl acetate from the aqueous phase in a pH range from 1–8. Also the partition coefficient obtained from extraction of the ethyl acetate fraction into $NaHCO_3$ was characteristic of a polar neutral compound. The possibility that the amino group was inactivated by a peptide linkage was excluded since after acid and alkaline hydrolysis no amine was recovered. Failure to form a semicarbizide demonstrates that it is not an aldehyde. It was readily acetylated, and from its mobility in the Bush "C" solvent system, it was again shown to be a polar substance. Since the ratio of ${}^{3}H/{}^{14}C$ was the same in this new metabolite as in the injected solution, the configuration of the α-carbon and β,β-hydrogen was retained. Its stability in alkaline solution suggests that it is most likely a 3-methoxy derivative of dopamine. All of the above findings indicate that this compound is 3-methoxy-4-hydroxyphenylethanol and that it is formed through a pathway analogous to that of 3-methoxy-4-hydroxyphenylglycol formation from norepinephrine[2]. The formation of this neutral compound is enhanced by iproniazid treatment as shown in Table I.

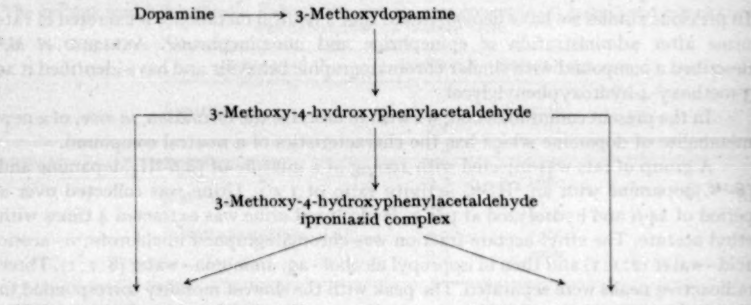
TABLE I

^{3}H- AND ^{14}C-ACTIVITY OF 3-METHOXY-4-HYDROXYPHENYLACETIC ACID AND OF THE NEUTRAL METABOLITE ISOLATED FROM THE URINE OF RATS TREATED WITH A MIXTURE OF [β,β-$^{3}H_{2}$]DOPAMINE AND [α-^{14}C]DOPAMINE

Compound	*Normal rats*			*Iproniazid-treated rats*[*]		
	total activity (counts/min) × 10^{3}		*ratio of $^{3}H/^{14}C$*	*total activity (counts/min) × 10^{3}*		*ratio of $^{3}H/^{14}C$*
	^{3}H	*^{14}C*		*^{3}H*	*^{14}C*	
3-Methoxy-4-hydroxyphenylacetic acid	72.75	55.2	1.3/1	42.62	33.6	1.2/1
Neutral metabolite	4.2	3.85	1.1/1	12.75	8.84	1.3/1

[*] The dose of iproniazid administered (60 mg/kg) only partially inhibited the metabolism of dopamine by monamine oxidase.

The following pathways are postulated as a possible explanation for the above findings:


Dopamine
3-Methoxydopamine
3-Methoxy-4-hydroxyphenylacetaldehyde
3-Methoxy-4-hydroxyphenylacetaldehyde
iproniazid complex
3-Methoxy-4-hydroxyphenylacetic acid
3-Methoxy-4-hydroxyphenylethanol

In this pathway, a 3-methoxy-4-hydroxyphenylacetaldehyde-iproniazid complex is proposed. This complex is similar to the type found by ZELLER[4] which results from the reaction of 1,2-dialkylhydrazines with aldehydes The formation of such a complex would stabilize the aldehyde stage and protect the aldehyde to some degree from oxidation to the corresponding acid. This would then enhance the biological formation of alcohol as demonstrated in the iproniazid-treated rats. The isolation of this compound from rat urine is now being carried out after administration of large doses of non-radioactive dopamine.

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Neurochemistry Laboratory, M. GOLDSTEIN
Department of Psychiatry and Neurology, A. J. FRIEDHOFF
New York University College of Medicine, New York, N.Y. (U.S.A.) S. POMERANTZ
C. SIMMONS

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The activation of the inducible enzyme, rat-liver tryptophan pyrrolase

Tryptophan pyrrolase has been shown to be an iron-porphyrin protein[1]; when rat-liver homogenates are centrifuged at 11,000 × *g*, it is found exclusively in the supernatant[2]. Using a slight modification of the assay method of Knox and Mehler[2], based on the kinetic measurement of kynurenine accumulation, we have confirmed that the centrifugal removal of nuclei or of nuclei and mitochondria from normal rat-liver homogenates do not affect the tryptophan pyrrolase activity. However, when the microsomal fraction was eliminated as well, by centrifuging at 105,000 × *g* for 50 min, only about one-third of the activity remained in the supernatant. Recombination of this soluble cell sap with either microsomes or mitochondria restored the original enzyme activity, although neither of these particulate fractions alone manifested any activity. It can be seen from Table I that boiling of the microsomes for 20 min did not destroy their stimulatory effect; ashed microsomes, however, were devoid of activity, indicating a heat-stable organic activator. The active factor could be extracted from microsomes by shaking in a pH 7.4 buffer at 37° for 30 min, but not by boiling. A protein-free HCl–acetone extract, known to contain iron-porphyrins[3], was nearly as active as the corresponding amount of intact microsomes. Ferriheme,

TABLE I

THE ACTIVATION OF LIVER-CELL-SAP TRYPTOPHAN PYRROLASE

Livers of Sprague-Dawley rats were homogenized in a Waring blendor in 7 vol. 0.14 *M* KCl containing 0.0025 *N* NaOH, and centrifuged at 12,000 × *g* for 20 min at 0°. The supernatant was centrifuged again at 105,000 × *g* for 50 min at 0° giving cell sap and microsomal fraction. The latter, where indicated, was resuspended in the assay mixture by gentle homogenization. For assay of the enzyme, the cell sap was incubated with 0.003 *M* L-tryptophan with additions as indicated, at 37° in a buffered medium with continuous shaking in air. Aliquots were taken for kynurenine estimation at intervals of 10–15 min for 105 min. The amount of untreated or treated microsomes added corresponded to twice the ratio of microsomes to cell sap in the liver.

Additions to cell sap	*Tryptophan pyrrolase activity (µmoles kynurenine/h/g liver)*
None	1.8
Intact microsomes	4.5
0.2 *N* phosphate buffer extract of microsomes	3.0
Boiled microsomes	4.2
Filtrate of boiled microsomes	2.0
Precipitate of boiled microsomes	3.5
Ashed microsomes	1.8
Acid-acetone extract of microsomes	4.2
Ferriheme (0.03 mg/ml)	3.8

prepared from commercially obtained hemin, also showed stimulatory action. It therefore seems quite possible that the active principle derived from microsomes is a metalloporphyrin.

The tryptophan pyrrolase activity of rat liver increases several-fold following the intraperitoneal injection of L-tryptophan[2]. It has now been observed that the elimination of microsomes from the 12,000 × *g* liver supernatant of these "induced" rats did not significantly reduce the tryptophan pyrrolase activity. A typical experiment, illustrated in Table II, shows that the addition of microsomes more than doubled the tryptophan pyrrolase activity of normal cell sap, whereas the enzyme activity of "induced" cell sap was unchanged. If cell sap of "induced" animals is diluted so as to give enzyme levels similar to those of normal cell sap, or if microsomes derived from "induced" rats are used, the results remain essentially the same.

TABLE II

THE EFFECT OF MICROSOMES ON THE TRYPTOPHAN PYRROLASE ACTIVITY OF NORMAL AND "INDUCED" RATS

The "adapted" rats received 1.82 ml of 5.08 % L-tryptophan suspension in 0.9 % saline per 100 g body weight, intraperitoneally, and were sacrificed 2 h later. Homogenates were treated as described in Table I. The microsomes were derived from normal livers. The ratio of microsomes to cell sap was twice in the liver.

Additions	*Tryptophan pyrrolase (µmoles kynurenine/h/g liver)*	
	normal cell sap	*"induced" cell sap*
None	1.8	8.4
Normal microsomes	6.5	8.8

These experiments suggest the possibility that under the usual assay conditions, tryptophan pyrrolase of normal-liver cell sap is unsaturated with respect to its activator, and, that during the process of substrate induction, the activator, which is a normal constituent of cytoplasmic granules, becomes available to the enzyme. Thus, the substrate induction of this enzyme may be due, at least in part, to an intracellular translocation of the activator.

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Department of Biochemistry and Medicine,
Columbia University College of Physicians and Surgeons,
New York, N.Y. (U.S.A.)

OLGA GREENGARD
PHILIP FEIGELSON[*]

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[*] Established Investigator, Health Research Council, New York, N.Y.

GEL FILTRATION OF PROTEINS, PEPTIDES AND AMINO ACIDS

JERKER PORATH

Institute o Biochemistry, University of Uppsala, Uppsala (Sweden)

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SUMMARY

1. Mixtures of proteins, peptides and amino acids can be fractionated by filtration through beds of dextran gel containing only small amounts of carboxylic groups.

2. Group separations are readily achieved. In highly cross-linked dextran proteins and large peptides move together ahead of amino acids. In dextran gels of low degree of cross-linking peptides and even proteins may be retained on the columns, so that a fractionation of substances within these groups may be obtained.

3. Basic peptides and amino acids move slowly through the gels in certain basic solvents such as 1 *M* pyridine and faster in acidic solvents such as 1 *M* acetic acid. For acidic peptides and amino acids the influence of the solvents mentioned appears to be the reverse of that for the basic compounds.

4. Aromatic substitution has a marked effect on the migration through the gels. The relative speed of dinitrophenylated amino acids is highly dependent on the buffer used. Such influence of the buffer was not noticed for phenylalanine, tyrosine and tryptophan, although these compounds are retarded to a different extent.

5. When the columns are properly prepared, symmetrical distribution of each compound is always obtained.

6. The column capacity is very high compared to other similar column methods (chromatography and zone electrophoresis).

7. The reproducibility is very good.

8. The gels are easily regenerated in the columns and may be used daily over a period of months without detectable deterioration.

INTRODUCTION

In a previous communication[1] it was reported that when a mixture is filtered through a column of ground dextran gel, substances of large molecular size tend to move at a faster rate than those of small size. If the difference in size between two substances is sufficiently large, a complete separation may be achieved.

The molecular sieve effect is presumably caused by steric hindrance which restricts the diffusion of the components into and through the gel phase to different extent. However, molecular sieving alone can not account for all the separation phenomena encountered in dextran gel filtration. If a substance is able to enter the gel phase sorption may also occur. The present study was made with the objective of obtaining qualitative information on the interaction of dextran gels with proteins, peptides, amino acids, and some of their derivatives under varying conditions. Further information about operational conditions of dextran gels, recoveries etc. may be found in communications by FLODIN[2] and GELOTTE[3].

MATERIALS AND METHODS

Dextran gels

Two types of cross-linked dextran, "dextran gel", labeled B 280 and B 197, have been used throughout this investigation. They were supplied by Pharmacia, Uppsala, Sweden and are characterized by different expansion capacities in aqueous solutions. (Similar gels under the trade name "Sephadex" can be obtained from Pharmacia.) Gels swollen in water are semitransparent and "from practical standpoint" completely insoluble. The gel B 280 has a true wet density of 1.06 g/ml[4] and a water regain of 5.5 g/g of dry wt. This implies that it is built up in a loose network structure. The gel B 197 on the other hand is a polymer of high degree of cross-linking, with a true wet density of 1.14 and a water regain of 1.9. Further information concerning general properties of dextran gels can be found in FLODIN's and GELOTTE's papers[2, 3].

The ground gels were sieved in dry state and the particles passing 100 but not 200 mesh sieves were collected. The particles were allowed to swell in a 1 % aqueous sodium chloride solution for 1 to 2 h after which they were sedimented to remove "fines". The gels were collected on filter, washed with distilled water and finally air dried. After lyophilization of 500 ml water or 1 *M* acetic acid washings from column I described below, solids could not be detected by visual inspection of the containers or by weighing after repeated lyophilization in small plastic bottles.

Substances: The substances used in this investigation were obtained as follows: Amino acids of analytical grade from Merck and Co. Rahway, N.J.; DNP-amino acids were prepared according to SANGER[5]; Oxidized glutathione of analytical grade and cytochrome *c* from Nutritional Biochemical Corporation, Cleveland, Ohio; Lysozyme Lot 78-77 from Sigma Chemical Company, St. Louis, Missouri; Polymyxin B-sulfate Lot A 5675 assaying 7430 units/mg from Mann Research Laboratories. Pepsin, twice crystallized, from Worthington Biochemical Corporation, Freehold, N. J.; Beef insulin crystalline, assaying 23 international units/mg of the lot (Butch No 2189) prepared at the request of the Commission of Proteins IUPAC; Human serum albumin electrophoretically homogeneous in phosphate–borate buffer of pH 8.4 and ionic strength 0.1 was prepared by zone electrophoresis as earlier described[6]; Bacitracin A was purified by charcoal chromatography and zone electrophoresis[7]; Phycocyanin and phycoerythrin were prepared from *Ceremium rubrum*[8]; Vasopressin and melanophore stimulating hormone (βMSH) from pig pituitaries[9, 10], Viscotoxin was a gift from Dr. G. SAMUELSSON, Institute of Pharmacy, Stockholm; Crude phthaloyl-γ-aminobutyryl-histidine was synthesized according to the method of TURNER[12]. Details of the preparation procedure will be published later[13]. Ferriporphyrin peptide was prepared as described by TUPPY AND PALÉUS[14].

Preparation of columns

All experiments were performed either on gel B280 or B197. The columns were packed as follows.

Column I: Air-dried gel B 280 (about 55 g) was suspended in 25 volumes of phosphate buffer of pH 6.8 and ionic strength μ 0.05. The thin suspension was transferred to a large funnel, provided with a stirrer, and connected with a 100 × 3 cm chromatography tube of glass containing a cotton plug at the bottom and filled with buffer solution. When a bed of a few centimeters had formed above the cotton plug the stopcock at the bottom of the column tube was opened and the gel grains were allowed to settle under liquid flow[2,15]. The final column measured 74 cm in height and was found with phycocyanin and albumin as test substances to possess a void volume of 122 ml in all buffers, except in borate solutions which yielded lower volume values.

Column II: A column bed 3 × 65 cm, of the gel B 197 (about 140 g dry gel) was prepared in a similar manner as Column I. The hold-up of phycocyanin and serum albumin was found to be 136 ml.

The columns could be washed with the buffer solutions desired (pH 2.4–9.3) without notable change of the outer dimensions of the beds. In alkaline solution the rate of flow was slower at a given hydrostatic pressure than in neutral or acid media. No deterioration of the gel has ever been noticed after frequent use for more than half a year.

Filtration technique

Where not otherwise stated, 0.5–20 mg of each substance, alone or in mixture, was dissolved in 3 ml of buffer solution. The solution was placed on top of the column and slowly filtered down. The rate of the subsequent elution with the buffer was usually adjusted so that 4–5-ml fractions were collected every 10 min. In some cases the columns were loaded with considerably more material. All experiments have been performed at room temperature.

Analytical methods

Absorption in visual and u.v. light was determined in Beckman spectrophotometer Model DU.

The ninhydrin reaction was performed according to MOORE AND STEIN[16].

Proteins and peptides were determined with the Folin method modified according to LOWRY *et al.*[17]. Histidine and phthaloyl-γ-aminobutyryl-histidine in 1 *M* pyridine solution was determined with PAULI-MACPHERSON's method[18] modified as follows:

The sample was adjusted to about pH 6.5 with 1 *M* HCl. To 0.05–0.1 ml of the sample 0.2 ml of sulfanilic acid reagent (1 % sulfanilic acid in 10 % HCl) and 0.2 ml 5 % sodium nitrate solution were added. The mixture was vigorously shaken for 1–2 min and left for 30 min. 1 ml 20 % sodium carbonate solution was added. After mixing the solution was diluted with 2 ml of water (or more if necessary) and read in Beckman spectrophotometer Model B at 490 mμ.

RESULTS

Influence of ionic strength

A series of filtrations on column I of easily detectable substances was performed in phosphate-buffers of pH 6.8. The R_F values are compiled in Table I. Under these conditions the effect of ionic strength is surprisingly small.

In basic buffers the void volumes of the gel columns are decreased as compared with those found at neutrality or in acid solutions. It was thought likely that this change would affect the filtration behaviour of the substances differently. Serum albumin, cytochrome *c* and lysozyme were studied on column I in buffers of different salt concentration but at the same pH (9.3). The results are shown in Table II. A striking effect was found in the case of lysozyme which moves comparatively much faster at high sodium salt concentration. In ammonium carbonate, however, the retardation is very strong even in 0.3 *M* solution.

TABLE I

R_F VALUES ON DEXTRAN GEL OF LOW DEGREE OF CROSSLINKING (B 280) IN PHOSPHATE BUFFERS OF pH 6.8 WITH DIFFERENT IONIC STRENGTH

μ	*Ileu*	*DNP-ileu*	*Lysozyme*	*Cytochrome c*	*Phycocyanin*	*Try*
0.01	0.35	0.27	0.55	0.69	1.00	0.24
0.05	0.34	0.28	0.60	0.71	1.00	0.25
0.20	0.35	0.27	—	0.70	1.00	0.24

TABLE II

R_F VALUES ON GEL B 280 IN VARIOUS BUFFERS OF pH 9.3

Buffer system	*Albumin*	*Cytochrome*	*Lysozyme*
0.3 *M* Ammonium carbonate	1.00	0.82	0.31
0.02 *M* Na-borate	1.00	0.87	0.29
0.02 *M* Na-borate + 0.2 *M* salicylate	—	0.87	0.62
0.02 *M* Na-borate + 0.5 *M* NaCl	1.00	—	0.74

Influence of pH

The sensitivity of filtration behaviour towards pH-changes was tested for some representative substances on column I. As seen from Table III the R_F values of glycine and isoleucine and their DNP-derivatives are almost independent of the pH of the elutrient. Cytochrome *c* is most strongly retained in the phosphate buffer of pH 6.8 and moves with a considerably higher relative speed in more acidic as well as in alkaline solutions. Lysozyme shows a steadily increasing retention with pH in the interval studied. Insulin follows a more complicated pattern which probably reflects shifts in association–dissociation equilibria of different polymers. In acid solutions,

TABLE III

R_F VALUES ON DEXTRAN GEL OF LOW DEGREE OF CROSSLINKING (B 280) IN BUFFERS OF VARIOUS pH

pH	*Ileu*	*Gly*	*DNP-ileu*	*DNP-gly*	*Insulin*	*Cytochrome c*	*Lysozyme*
0.7 (1 *M* HAc)	—	0.33	—	0.25	0.53	0.90	0.68
2.4 (1 *M* DCA*)	—	0.33	—	0.27	0.61	—	—
4.0 (acetate)**	0.33	0.33	0.26	0.26	0.61	0.77	0.61
6.8 (phosphate)**	0.34	0.33	0.28	0.27	0.90	0.71	0.60
8.4 (phosphate-borate)[2]	0.34	0.32	0.28	0.27	0.80–0.61	0.90	0.44

* Dichloroacetic acid.

** Ionic strength 0.05.

insulin gives a single sharp peak, whereas in the phosphate–borate buffer of pH 8.4 a very broad distribution is obtained with a well developed maximum at $R_F = 0.80$ and a hidden maximum at $R_F = 0.64$.

Influence of gel structure

A few substances were filtered through columns I and II in phosphate buffer at pH 6.8 ($\mu = 0.05$). The results (see Fig. 1 and Table IV) show how the degree of cross-linking affects the filtration behaviour. It is particularly noteworthy that cytochrome *c* moves with almost the same rate as phycocyanin on the tightly cross-linked gel B 197 whereas the two proteins on the loose gel B 280 can easily be separated.

A synthetic mixture of viscotoxin and phycocyanin was fractionated on columns I and II in phosphate buffer of pH 6.8 ($\mu = 0.05$) under as similar conditions as possible. The distributions obtained are shown in Figs. 3 and 4.

TABLE IV

R_F VALUES OBTAINED IN PHOSPHATE BUFFER OF pH 6.83 AND IONIC STRENGTH 0.05 WITH TWO GELS OF DIFFERENT DEGREE OF CROSSLINKING

Gel	*Try*	*Ileu*	*DNP-ileu*	*Lysozyme*	*Cytochrome c*	*Phycocyanin*
B 280	0.25	0.34	0.28	0.60	0.71	1.00
B 197	0.22	0.50	0.34	—	0.96	1.00

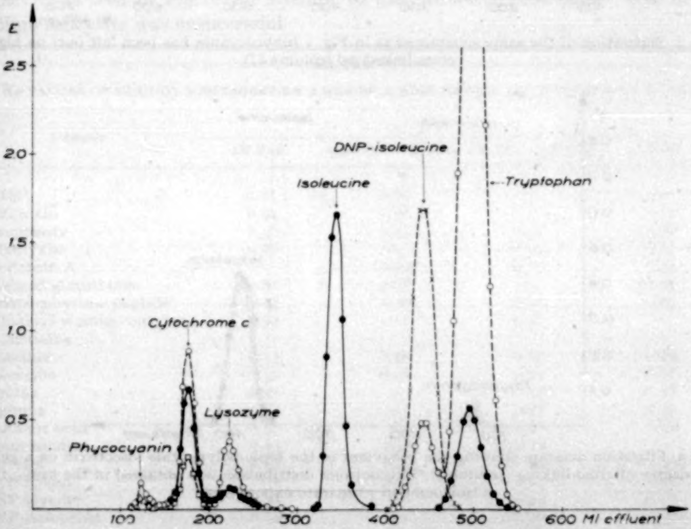

Fig. 1. Filtration diagram of an artificial mixture of selected substances. Filtration was made on a gel of low degree of cross-linking (column I) in phosphate buffer of pH 6.8 and ionic strength 0.05. △—△ O.D. at 615 $m\mu$. ○—○ O.D. at 280 $m\mu$. □—□ O.D. at 550 $m\mu$. ×—× O.D. at 360 $m\mu$. ●—● Ninhydrin color. The symbols have the same meaning in the proceeding figures.

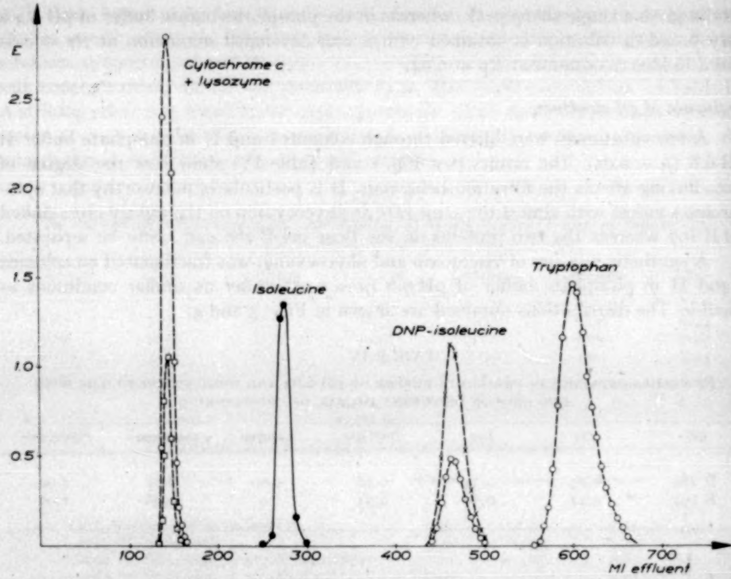

Fig. 2. Separation of the same substances as in Fig. 1 (phycocyanin has been left out) on highly cross-linked gel (column II).

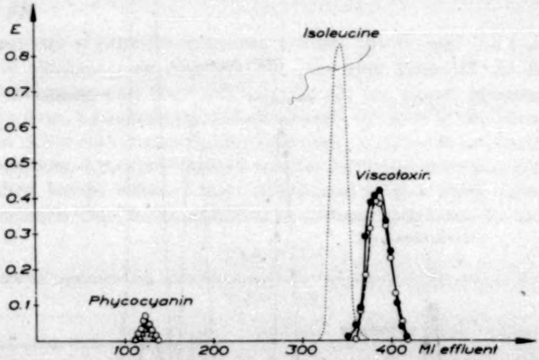

Fig. 3. Filtration diagram showing the behaviour of the basic polypeptide viscotoxin on a gel of low degree of cross-linking (column I). Phycocyanin distribution was obtained in the same, that of isoleucine in a separate experiment.

Influence of the aromatic character

It may be suspected that aromatic substitution generally increases the retention since in the experiments hitherto referred tryptophan has moved slower than iso-

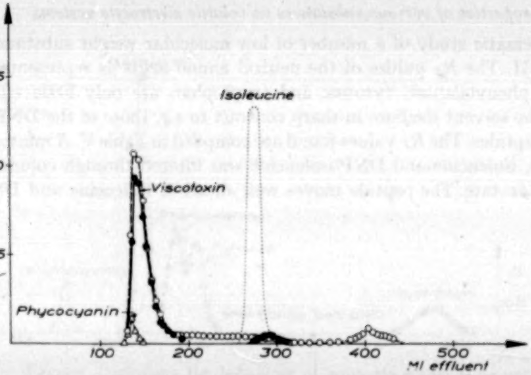

Fig. 4. Filtration diagram showing that viscotoxin on column of a highly cross-linked gel moves ahead of isoleucine and only slightly slower than phycocyanin.

leucine and the DNP-glycine and DNP-isoleucine slower than their parent amino acids. To test this hypothesis, a synthetic mixture of isoleucine, phenylalanine, tyrosine and tryptophan, and albumin (as reference) was filtered through column II in 0.02 *M* sodium borate (pH 9.3). A definite separation of all components was achieved as seen in Fig. 6. An attempt to use "aromatic saturation" with 0.2 *M* sodium salicylate was unsuccessful.

TABLE V

R_F VALUES OF VARIOUS SUBSTANCES ON A GEL OF A HIGH DEGREE OF CROSSLINKING (B 197)

Substance	*Buffer system*			
	1M HAc	*0.3M Py + y.1M HAc*	*1M Py + 0.05M HAc*	*1M Py*
β MSH	0.81	0.80	—	—
Viscotoxin	0.94	0.96	0.70	0.15
Vasopressin	0.56	0.55	0.51	0.30
Polymyxin	0.86	—	0.65	0.15
Bacitracin A	—	0.63	0.70	0.75
Oxidized glutathione	0.61	0.66	—	0.99
Ferriporphyrin-*c* peptide	0.53	0.70	0.80	0.95
Phthaloyl-γ-amino butyryl-histidine	0.50	—	0.57	—
Histidine	0.53	0.50	0.50	0.49
Isoleucine	0.52	0.51	0.50	0.50
Glycine	0.51	—	0.50	0.48
Arginine	0.52	—	0.41	0.30
Glutamic acid	0.51	—	0.48	0.60
Phenylalanine	0.45	0.45	0.47	—
Tyrosine	0.40	0.39	0.39	0.39
Tryptophan	—	0.27	0.27	0.27
DNP-glycine	0.26	—	0.34	0.77
DNP-isoleucine	0.28	—	0.36	0.77
DNP-tryptophan	0.09	—	0.21	0.75
Na-phosphate	—	0.45	—	—
Na-borate	—	0.40	—	—

Filtration properties of various substances in volatile electrolyte systems

A systematic study of a number of low molecular weight substances was made on column II. The R_F values of the neutral amino acids as represented by glycine, isoleucine, phenylalanine, tyrosine and tryptophan are only little affected by the nature of the solvent medium in sharp contrast to *e.g.* those of the DNP-amino acids and basic peptides. The R_F values found are compiled in Table V. A mixture of oxidized glutathione, isoleucine and DNP-isoleucine was filtered through column II in 0.1 *M* pyridinium acetate. The peptide moves well ahead of isoleucine and DNP-isoleucine (Fig. 5).

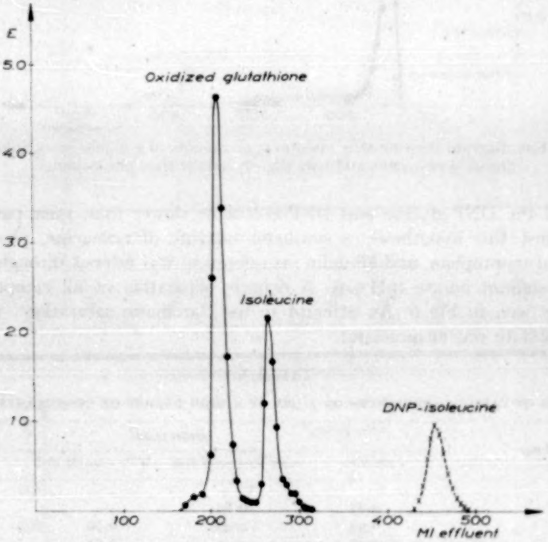

Fig. 5. Separation of oxidized glutathione, isoleucine and DNP-isoleucine on a highly cross-linked gel (column II) in 0.1 *M* pyridinium acetate.

Fractionation of phthaloyl-γ-aminobutyryl-histidine and histidine as a model experiment for gel filtration in organic synthetic work

The following three experiments were carried out on the tight gel B 197 on Column II with different loads of a partly purified reaction product containing phthaloyl-γ-aminobutyryl-histidine and histidine.

1. 100 mg of the mixture was dissolved in 6 ml of 1 *M* acetic acid and pressed into the Column II prewashed with the same solvent. The filtration was performed at a rate of 4 ml/every 5 min. Histidine was located by the ninhydrin reaction (0.1 ml aliquots per fraction) and phthaloyl-γ-aminobutyryl-histidine by the u.v. absorption at 295 mμ. Histidine appeared ahead of phthaloyl-γ-aminobutyryl-histidine but not completely separated from the latter (Fig. 7).

2. An amount of 0.85 mg of the mixture dissolved in 1 ml 1 *M* pyridine was

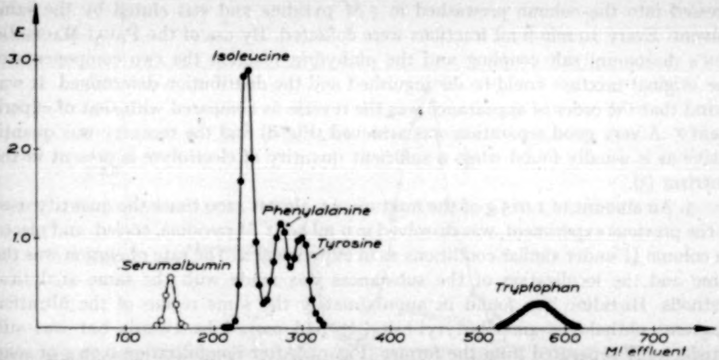

Fig. 6. Filtration diagram illustrating the behaviour of aromatic amino acids onhighly cross-linked gel (column II) in 0.02 *M* sodium borate. Serum albumin was used as a reference.

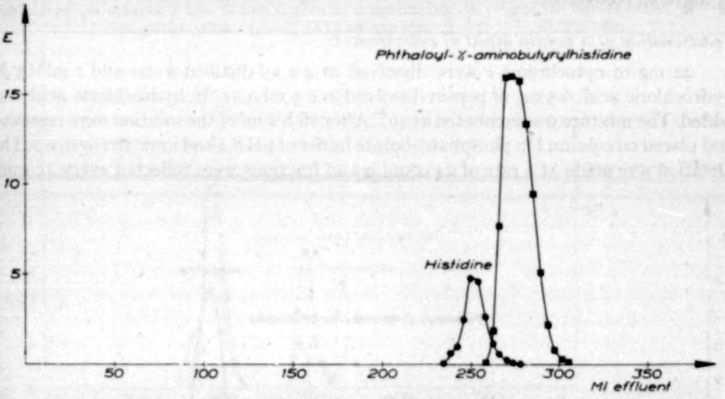

Fig. 7. Filtration diagram of 100 mg of a partly purified intermediate in the synthesis of γ-aminobutyrylhistidine. Filtration was made in highly cross-linked gel (column II) in acetic acid. ●—● Ninhydrin colour. ■—■ O.D. at 295 mμ.

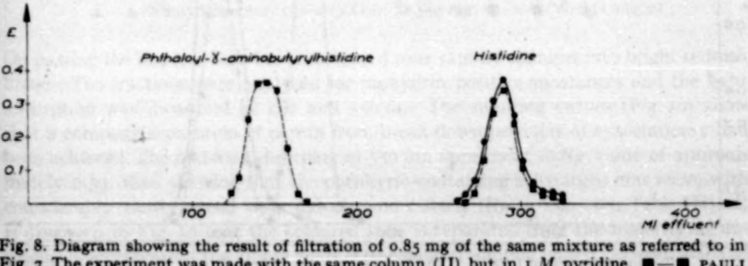

Fig. 8. Diagram showing the result of filtration of 0.85 mg of the same mixture as referred to in Fig. 7. The experiment was made with the same column (II) but in 1 *M* pyridine. ■—■ PAULI reaction colour.

pressed into the column prewashed in 1 *M* pyridine and was eluted by the same solvent. Every 10 min 6-ml fractions were collected. By use of the PAULI-MACPHERSON's diazonium salt coupling and the ninhydrin reaction the two components of the original mixture could be distinguished and the distribution determined. It was found that the order of appearance was the reverse as compared with that of experiment 1. A very good separation was achieved (Fig. 8) and the recovery was quantitative as is usually found when a sufficient quantity of electrolyte is present in the elutrient (3).

3. An amount of 1.015 g of the mixture, *i.e.* almost 1200 times the quantity used in the previous experiment, was dissolved in 6 ml hot 1 *M* pyridine, cooled, and placed on column II under similar conditions as in experiment 2. The rate of elution was the same and the localization of the substances was made with the same analytical methods. Histidine was found in approximately the same region of the filtration diagram; phthaloyl-γ-aminobutyryl-histidine had moved backwards but was still satisfactorily separated from the former (Fig. 9). After lyophilization 0.99 g of solid substances was obtained. The samples taken for analysis can account for the deficit in material recovered.

Fractionation of a pepsin digest of cytochrome c

42 mg of cytochrome *c* were dissolved in 4.2 ml distilled water and 1 ml 0.3 *M* hydrochloric acid. 6.5 mg of pepsin dissolved in 0.5 ml 0.01 *M* hydrochloric acid was added. The mixture was incubated at 40°. After 26 h 2 ml of the solution were removed and placed on column I in phosphate–borate buffer of pH 8.4 and ionic strength 0.1. The filtration was made at a rate of 24–25 ml/h and fractions were collected every 10 min.

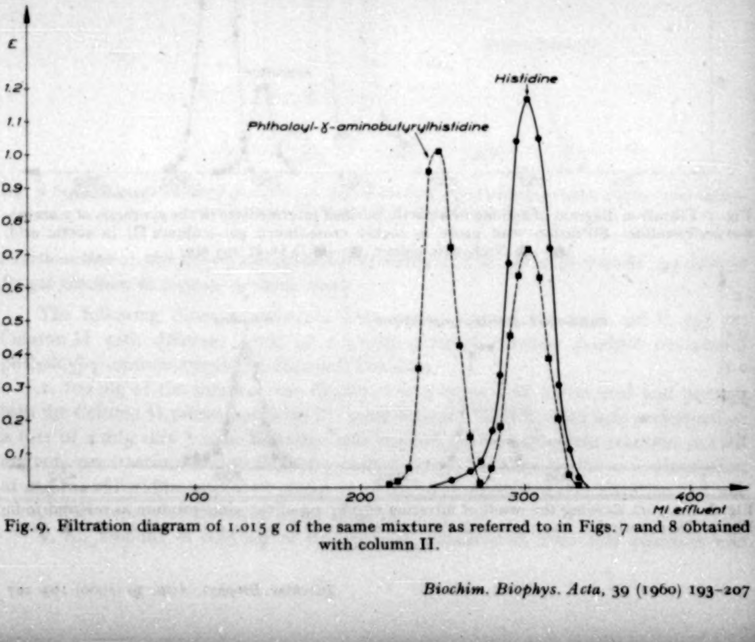

Fig. 9. Filtration diagram of 1.015 g of the same mixture as referred to in Figs. 7 and 8 obtained with column II.

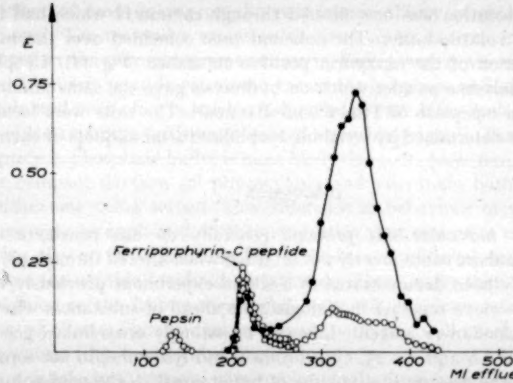

Fig. 10. Filtration diagram of a peptic digest of cytochrome *c*. After digestion the acid sample was placed on column I (gel of low degree of cross-linking) in phosphate borate buffer. ●—● Ninhydrin colour. □—□ O.D. at 550 mμ. O—O O.D. at 280 mμ.

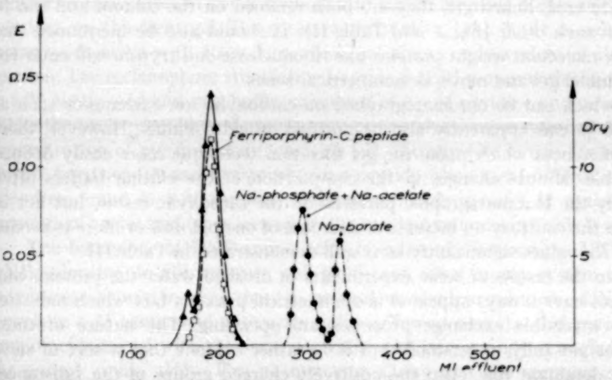

Fig. 11. Diagram illustrating removal of non-volatile salts from the peptide in the colored zone of Fig. 10. The filtration experiment was made on column II in pyridine–acetic acid buffer. ▲—▲ Ninhydrin color. □---□ O.D. at 550 mμ. ●-·-● Weight mg/ml.

On passing the bed the dark brown coloured zone rapidly changed into bright reddish brown. The fractions were analyzed for ninhydrin positive substances and the light absorption was measured at 280 and 550 mμ. The resulting curves (Fig. 10) show that a complete separation of pepsin from break-down products of cytochrome *c* had been achieved. The material absorbing at 550 mμ appears at a R_F value of approximately 0.59, thus showing that the porphyrin-containing substances now move with considerably slower speed than cytochrome *c* itself ($R_F = 0.90$; see Table III). It is also seen in Fig. 10 that the coloured zone is separated from the majority of uncoloured peptides and the amino acids. The coloured solution eluted between 200 and 225 ml was lyophilized and dissolved in 3 ml of a buffer of 0.3 *M* pyridine and 0.1 *M* acetic acid. This solution was now filtered through column II which had been prewashed with the volatile buffer. The coloured zone coincided over the major part with the distribution of the ninhydrin positive substance (Fig. 11). Lyophilization yielded a brown salt-free powder which on hydrolysis gave the same amino acids as the ferriporphyrin *c*-peptide of TUPPY AND PALÉUS[14]. The salts were localized and their distributions determined by weighing lyophilized 2-ml aliquots of each fraction.

DISCUSSION

Because of their molecular size proteins generally do not penetrate or adsorb on the gel grains and are consequently not retarded when filtered through gel columns. This has strikingly been demonstrated in a serum experiment previously reported[1], where all proteins move together in a single zone ahead of substances which do not react with the Folin-Lowry reagent. Likewise on strongly cross-linked gels in phosphate buffer of pH 6.8 and μ 0.05, cytochrome *c* and lysozyme do not separate and emerge just after the passage of a volume of buffer equal to the void volume of the bed. As expected molecules of intermediate size may be retarded under suitable conditions. When lysozyme and cytochrome *c*, for instance, are filtered through a bed of a weakly cross-linked gel, they are both retained on the column and can be separated from each other (Fig. 1 and Table II). It should also be mentioned that other basic low molecular weight proteins like ribonuclease and trypsin will enter the weakly cross-linked gel and move as symmetrical zones.

Proteins which can be chromatographed on carboxylic ion exchangers such as Amberlite IRC-50 can apparently also be purified on gel columns. However, there are marked differences which make the gel filtration technique more easily defined and reproducible. Minute changes in the composition of the eluting buffers often alter drastically the chromatographic patterns on the carboxylic resins, but not so on the gels. On the contrary an increase or decrease of one pH unit or more is needed to change the R_F values significantly as is well demonstrated in Table III.

Apart from the results of some experiments in distilled water the proteins and other substances have always appeared in symmetrical peaks, a fact which indicates that perfectly reversible exchange processes are operating. The nature of these processes is not yet fully understood, but it is rather evident that a sort of sieve action plays a dominant role. Also the positively charged groups of the substances influence the filtration behaviour since basic peptides and amino acids as well as the basic proteins appear later in the eluate than do neutral substances of comparable molecular size (see for instance Table V). It should be noted (Table III) that the retention of cytochrome *c* passes a maximum while lysozyme is progressively more retarded with increasing pH of the buffer. Increase in salt concentration enhances the relative speed of a lysozyme zone, an effect which is particularly pronounced in basic solution (Table II). The stronger sorption of lysozyme, viscotoxin, polymyxin, vasopressin and arginine (Tables I and V) can probably not be explained only on the assumption that ionic forces are involved in the gel–solute interaction. It is of interest that LATHE AND RUTHVEN[19] found that retardation of basic substances on starch is greatest in acid media. Obviously starch acts also as a cation exchanger. This is a noteworthy difference in behaviour of the two materials because the dextran gels and starch often show a remarkable resemblance in sorption properties.

The effect of cross-linking on the penetration ability of proteins has already been discussed. The molecular exclusion or sieve action is even more convincingly demonstrated in the chromatograms shown in Figs. 3 and 4. On the highly swollen gel (B 280) the basic peptide viscotoxin enters the gel phase and appears far behind phycocyanin and even after isoleucine although the viscotoxin molecule is of a rather large size since 11 amino acids are obtained upon hydrolysis[11]. The strong retention of this peptide in phosphate buffer is most likely due to its basic nature. When filtered through a compact dextran gel phycocyanin and viscotoxin both appear together without either one being sorbed. This difference in behaviour of viscotoxin on the two gels can only be explained on the assumption that molecular exclusion or molecular sieving play an important role. Fig. 5 shows an additional example of molecule sieving. Here the peptide (oxidized glutathione) moves faster than isoleucine.

In addition to the factors mentioned, aromatic character sometimes gives rise to unexpected effects. Substitution in the aromatic ring will exert a great influence on the R_F values (Fig. 6 and Table V).

Since a satisfactory removal of nonvolatile salts from solutions of low molecular weight substances generally presents a difficult problem, it is desirable to perform purification of such compounds in a volatile buffer system. R_F values found for a number of substances of small or moderate size in aqueous acetic acid and pyridine solutions on the tight gel (B 197) are tabulated in Table V. It is seen that neutral amino acids move with R_F values which are almost independent of the buffer composition. The melanophore stimulating peptide β-MSH moves with the same R_F value in 1 *M* acetic acid and in a buffer composed of 0.3 *M* pyridine and 0.1 *M* acetic acid. While the basic peptides and arginine are progressively more retarded with increasing concentration of pyridine and increasing pH, the opposite is true for oxidized glutathione, bacitracin A and ferriporphyrin *c* peptide. Very great difference in R_F values are often found in 1 *M* pyridine and buffers made up of 1 *M* pyridine and small amounts of acetic acid (as is seen when the two last columns in Table V are compared).

The behaviour of DNP-amino acids is extremely interesting. DNP-glycine and DNP-isoleucine do not separate well from each other in any system tried but they move more slowly in acid than in neutral or alkaline solutions. DNP-tryptophan travels in 1 *M* acetic acid with a R_F value as low as 0.09. In 1 *M* pyridine all DNP-amino acids travel with the same R_F value and interestingly enough faster than do the parent amino acids. This phenomenon shows that the sorption is strongly dependent on the electron distribution over the aromatic rings. These few examples indicate that gel filtration may provide a very valuable technique for the fractionation of water soluble aromatics.

The capacity of the gel columns is well demonstrated in the experiment where more than 1 g of impure phthaloyl-γ-amino-butyrylhistidine was placed on column II (Fig. 9). Ordinary liquid–liquid partition chromatography on a column of comparable size would certainly not give such well defined, almost symmetrical distribution peaks. At a much lower load, however, the two components of the mixture are better separated (Fig. 8). This is probably due to the larger contribution of the peptide itself, to the ionic strength of the solution in the former case and to the fact that the movement of the peptide in pyridine is more sensitive to such changes in the medium than is the histidine. In 1 *M* acetic acid the order of the two components of the mixture is opposite to that obtained in 1 *M* pyridine. These experiments demonstrate clearly a new promising means of purification of reaction mixtures on the ordinary laboratory scale as well as on the microscale.

Natural mixtures can easily be separated into a fraction containing proteins and other large molecular size substances and one or several fractions of simpler compounds. The proteins are very sharply separated from free amino acids. The protein peak may of course contain substances of small size that are strongly associated with the proteins. Reversible association–dissociation processes can often be utilized in gel filtration techniques to achieve a very efficient purification in two consecutive operations. This will be demonstrated elsewhere for certain hypophyseal hormones.

In sequential analysis, isolation of biologically active fragments from enzymes etc. there is need for effective methods that fractionate according to molecular size. It appears that dextran gels may very well be suited for such applications. Proteolytic enzymes can usually be removed from the digestion products and in the same operation the latter are more or less effectively separated. Fig. 10 may give an indication of the potentiality of dextran gel filtration. From a peptic digest of cytochrome *c* the porphyrin containing substances, chiefly, consisting of ferriporphyrin *c* peptide, have been freed of pepsin and the bulk of uncoloured peptides. The highly cross-linked gel was chosen since it was thought that this gel should give the largest difference in the R_F values between cytochrome *c* and its porphyrin-containing degradation products.

Finally, Fig. 11 illustrates the use of gel chromatography as a means of changing buffer systems. By choosing a volatile electrolyte, in this case pyridine–acetic acid buffer, the substance to be further investigated can be freed of salt by evaporation. This procedure is in fact very satisfactory and versatile and can very often be employed as can be realised by comparing the R_F values of sodium phosphate and borate with those of other substances in Table V. The list of useful non-volatile buffers that can be removed by filtration through a gel column in volatile salt solution can be extended with aromatic salts such as salicylate (see also ref.[2,3]). Metal salts may sometimes offer certain advantages over volatile buffers in ion exchange chromatography, zone electrophoresis, precipitation and other purification procedures. Thus the problem of desalting is often encountered in preparative work. If there is a very high demand regarding completeness and recovery there are few real satisfactory methods available for removal of such non-volatile from mixtures with peptides, amino acids, nucleotides etc. Gel filtration seems to offer an almost ideal solution to many desalting problems.

Notes concerning mechanism

The liquid inside and outside the gel may have different affinities towards the solutes and thus form a basis for liquid–liquid partition chromatography. However, the size of the solute molecules and the structure of the gel together have such a profound influence on the migration behaviour that it is not easily conceived how partition between two aqueous phases alone should be responsible for the separations. It appears much more likely that the discrimination with respect to molecular size can be attributed to the steric hindrance that different solute molecules encounter when passing into the gel phase. Very likely the retention of a substance on a granular gel column is dependent on the thickness of the gel layer which the molecules are able to penetrate[19]. While the entire gel phase (except possibly the hydrate shell of the gel proper[2,3]) is accessible to small molecules, large molecules interact only with the surface or the outermost layer of the gel grains.

With the two gels used in this investigation the corresponding lower limits of R_F values have been found to be approximately 0.50 and 0.33 respectively, if aliphatic amino acids such as glycine or isoleucine are assumed not to interact specifically with the gel (see Tables I, III, IV and V). Substances of intermediate molecular size will move with R_F values between 1.0 and the lower limits just referred to. The possibility of obtaining satisfactory separation within these R_F ranges appears to be greater in the case of gel filtration than in partition chromatography. It is evident, however, that the maximum efficiency of molecular sieving is achieved if the gel is so selected with respect to its degree of cross-linking that it fits the molecular dimensions of the substance to be purified. This possibility of variation offers great promise and makes the method particularly attractive.

R_F values have often been found which are not in accordance with the assumption that purely molecular sieving is responsible for the relative migration rates in gel beds. Both higher and lower R_F values than expected have been found. More fundamental research is necessary to reveal the mechanism involved in these positive and negative sorption phenomena.

The comparatively large capacity of dextran gel columns and the high flow rates that can be used without causing serious distortion and broadening of the moving zones are also indications that fractionation by gel filtration is based upon principles different from that of ordinary liquid–liquid partition chromatography.

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THE DIFFUSION CONSTANTS OF RABBIT, GUINEA-PIG AND RAT ANTIBODIES TO A SINCLE COMMON ANTIGEN

A. POLSON AND D. DEEKS
C.S.I.R./U.C.T. Virus Research Unit, Department of Pathology,
University of Cape Town (South Africa)

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SUMMARY

The diffusion constants of the precipitin antibodies prepared in rabbits, rats and guinea-pigs against the haemocyanin of *Burnupena cincta* have been determined by the gel-diffusion technique using the diffusion constant of the haemocyanin as reference standard. This work was done in an attempt to confirm the validity of the assumption in previous work, that the precipitating antibodies derived from the rabbit have the same diffusion constant as tetanus antitoxin derived from the horse. It was found that the diffusion constants of these precipitin antibodies are on the average appreciably higher than those given by other authors for gamma globulin and in this respect are confirmatory of the findings of LARGIER[4] if the diffusion constants determined by the present technique be regarded as being within 5 % of the true value.

INTRODUCTION

A modification of the gel-precipitation technique of OAKLEY AND FULTHORPE[1] by POLSON[2], is being applied in this laboratory to the estimation of diffusion constants and the concentration of antigens. In the plant virus field this technique has proved most useful (VAN REGENMORTEL[3]).

The diffusion constants of antigens are calculated from the equation:

$$D_g = D_b \frac{x_g^2}{x_b^2} \qquad (1)$$

where D_g and D_b denote the diffusion constants of the antigen and antibody respectively, and x_g and x_b the distances of the precipitin band from the antigen and antibody menisci, when the two reactants are present in their separate compartments in optimal proportions. The method for determining the optimal proportions of the reactants and for producing plane agar–solution interfaces has been described in detail in previous publications (POLSON[2]).

D_b in Eqn. (1) has been assumed to be constant and equal to $4.81 \cdot 10^{-7}$ cm^2/sec, a figure given by LARGIER for horse tetanus antitoxin. This diffusion constant is approximately 10 % higher than the usual figure quoted in the literature for gamma globulin.

A very likely explanation for this difference was offered by LARGIER[4] who found that his antitoxin was completely free of macroglobulin as shown by ultracentrifugation and suggested that this might not be the case with antisera investigated by other workers. In common with the laboratory practice of producing precipitating antibodies against antigens by inoculation of small animals, it was decided to verify LARGIER's figure by the examination of the diffusion rates of the precipitating antibodies derived from the guinea pig, the rat and the rabbit against a single antigen.

EXPERIMENTAL

2 rabbits, 2 guinea-pigs and 2 rats were immunized with the haemocyanin of *Burnupena cincta* (referred to in previous papers as *Caminella cincta*) by the subcutaneous route. After 6 injections the rabbits and guinea-pigs produced sera containing high titres of precipitating antibodies, while the rats showed a poor response even after 12 injections. The rat serum, however, proved suitable for use in gel-precipitin tests.

In Fig. 1 a photograph of the gel-precipitin apparatus is given showing the bands of precipitate in process of formation between the haemocyanin and rabbit antiserum. In Figs. 2–4 the results of gel-precipitin tests are given graphically. In each diagram the oblique lines indicate the variation of precipitin band position relative to the

Fig. 1. Photograph of the precipitin bands formed by different dilutions of *Burnupena cincta* haemocyanin against constant antiserum derived from the rabbit. Note the variation of precipitin band width with dilution of the antigen.

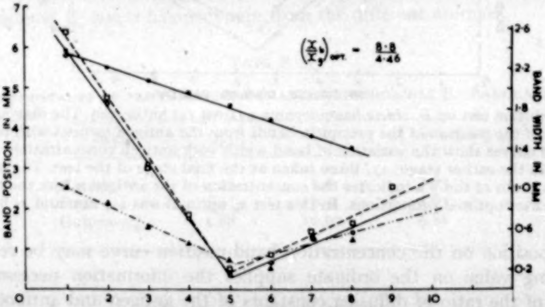

Fig. 2. Gel diffusion test on *Burnupena cincta* haemocyanin against rabbit antiserum. The diagonal line shows the variation of the position of the precipitin bands from the antigen menisci with concentration. The V shaped curves show the variation of band width with antigen concentration; ▲, measurements taken in the early stage; □, those taken at the final stage of the test. The point of intersection of the limbs of the V's indicates the concentration of the antigen where the two reactants are in exact optimal proportions. In this test x_g optimal was 4.46 mm and x_b 8.8 mm.

antigen menisci. The V-shaped lines indicate the variation of band width with antigen concentration at different times after commencement of the experiments. The point of intersection of the two limbs indicates the exact dilution where the two reactants are present in optimal proportions for the occurrence of precipitin reactions. Using this figure the position on the concentration band-position curve may be read and the corresponding value on the ordinate supplies the information necessary for the calculation of the ratio of diffusion constants of the antigen and antibody.

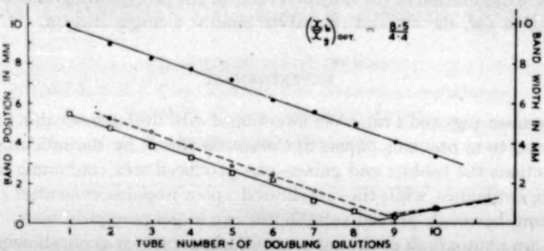

Fig. 3. Gel diffusion test on *Burnupena cincta* haemocyanin against guinea-pig antiserum. The diagonal line shows the variation of the position of the precipitin bands from the antigen menisci with concentration. The V shaped curves show the variation of band width with antigen concentration; ○, measurements taken in the early stage; □, those taken at the final stage of the test. The point of intersection of the limbs of the V's indicates the concentration of the antigen where the two reactants are in exact optimal proportions. In this text x_g optimal was 4.88 mm and x_b 10.02 mm.

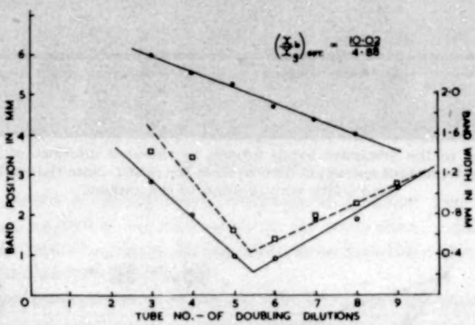

Fig. 4. Gel diffusion test on *B. cincta* haemocyanin against rat antiserum. The diagonal line shows the variation of the position of the precipitin bands from the antigen menisci with concentration. The V shaped curves show the variation of band width with antigen concentration; □, measurements taken in the earlier stage; ○, those taken at the final stage of the test. The point of intersection of the limbs of the V's indicates the concentration of the antigen where the two reactants are in exact optimal proportions. In this test x_g optimal was 4.4 mm and x_b 8.5 mm.

The diffusion constant of *B. cincta* haemocyanin has been determined by the Lamm scale method in which a new type of diffusion cell was used which made it possible to do four different diffusion experiments simultaneously (POLSON[5]).

Different concentrations of haemocyanin were diffused in $M/15$ phosphate buffer of pH 6.8. In Fig. 5 are given a set of diffusion gradient curves obtained after 141 h at 20°. The diffusion constants were calculated by the inflection point method. In Table I are given the values at different intervals of diffusion.

TABLE I

DIFFUSION CONSTANTS OF *B. cincta* HAEMOCYANIN CORRECTED FOR DIFFUSION IN WATER AT 20°

Time *h*	*Diffusion constants* × 10^7 *cm²/sec at concentration*			
	0.25%	*0.5%*	*0.75%*	*1.0%*
46	1.23	—	—	—
71.66	1.21	1.26		
141.0	1.24	1.23	1.26	1.25

Avarage $1.24 \cdot 10^{-7}$ cm²/sec

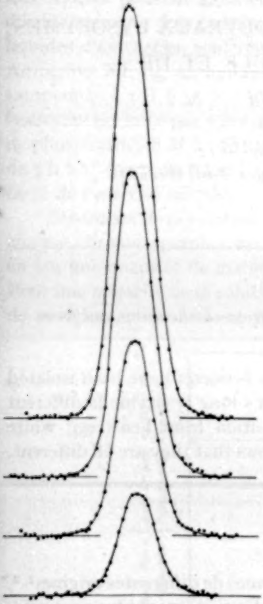

Fig. 5. Diffusion gradient curves obtained on 0.25, 0.5, 0.75 and 1.0 % solutions of *B. cincta* haemocyanin with the 4 compartment diffusion cell.

In Table II the results are given for the diffusion constants of the precipitating antibodies against *B. cincta* haemocyanin from the different animals.

TABLE II

DIFFUSION CONSTANTS OF THE PRECIPITATING ANTIBODIES AGAINST *B. cincta* HAEMOCYANIN

Animal	x_g	x_b	$D_b \cdot 10^6$ *cm²/sec*
Rabbit	4.46	8.8	4.83
Rat	4.4	8.5	4.63
Guinea-pig	4.88	10.02	5.21

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Biochim. Biophys. Acta, 39 (1960) 208–211

PURIFICATION ET ÉTUDE COMPARÉE DE NOUVEAUX LYSOZYMES; EXTRAITS DU POUMON DE POULE ET DE *NEPHTHYS HOMBERGI*

PIERRE JOLLÈS ET SIMONE ZUILI

Laboratoire de Chimie biologique, Faculté des Sciences, Paris (France)

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SUMMARY

Purification and comparative study of new lysozymes from chickenlung and from Nephthys hombergi

Two new lysozymes from hen's lung and from *Nephthys hombergi* have been isolated and obtained in a chromatographically pure state. Hen's lung lysozyme is different by its chromatographic behaviour and by its composition from hen's egg white lysozyme. A comparison between all these lysozymes shows that they are all different.

INTRODUCTION

Au cours de nos recherches sur la comparaison des lysozymes de différentes origines[1,2], deux nouveaux agents lysants ont été préparés à l'état chromatographiquement pur: le lysozyme de poumon de poule et le lysozyme de *Nephthys hombergi* (annélide polychète[3]). Le lysozyme de poumon de poule a été isolé en vue de comparer deux lysozymes provenant de deux organes différents d'un même animal (oeuf de poule et poumon de poule) et le lysozyme de *Nephthys hombergi* en vue de comparer un lysozyme provenant d'un invertébré avec les différents lysozymes de vertébrés déjà connus[1,4-6]. Ces enzymes ont été purifiés en soumettant à des chromatographies sur Amberlite XE-64 un matériel primaire obtenu dans des conditions particulières à chacun des lysozymes.

PARTIE EXPÉRIMENTALE

Activité lysante: L'activité lysante est mesurée vis-à-vis de suspensions d'une poudre acétonique de *Micrococcus lysodeikticus*, comme il a été indiqué précédemment[4].

Dosage des protéines totales: La concentration en protéine est déterminée par la méthode de Folin-Ciocalteu, selon le procédé indiqué par SUTHERLAND *et al.*[7].

Lysozyme du poumon de poule

Préparation du matériel primaire: Un poumon de poule pèse en moyenne 1.5 g et contient de 100 à 250 μg d'enzyme lysant par g d'organe frais (activité exprimée en µg de lysozyme d'oeuf). Les poumons doivent être prélevés immédiatement après que l'animal ait été tué et saigné, et il convient d'opérer dans une chambre froide. Les organes peuvent alors être conservés à —20° dans l'acétone contenant 0.75 % d'acide acétique. Les poumons sont broyés et extraits 4 ou 5 fois avec de l'eau. Les liquides d'extraction sont réunis et la protéine active est intégralement adsorbée sur Amberlite XE-64, en agitant pendant 3 h le liquide avec la résine préalablement tamponnée à pH 6.55 par une solution tampon de phosphates 0.2 *M*. L'élution du lysozyme est faite par 5 lavages successifs de la résine à l'aide d'une solution tampon de phosphates 0.8 *M* à pH 6.55. On élue ainsi 70 % de l'activité initiale. Après dialyse de 5 h à 2° (perte 10 %) et lyophilisation, on obtient "le matériel primaire" contenant 60 % de l'activité initiale.

Séparation de la substance active par chromatographie sur colonne d'Amberlite: Pour chaque chromatographie sur colonne d'Amberlite XE-64 (de 15 × 1 cm), on a mis en jeu une quantité de matériel primaire correspondant à 1 mg de substance active. Pour une molarité de la solution tampon de phosphates de 0.2 *M* à pH 6.40, 10 à 20 % de protéine active ne sont pas retenus et passent simplement à travers la colonne avant la mise en route du gradient; 80 % restent fixés sur la colonne et ne peuvent être élués qu'en élevant graduellement la molarité de la solution tampon à 0.6 *M* (Fig. 1). Il a été ainsi possible de caractériser deux fractions actives I et II dans le poumon de poule.

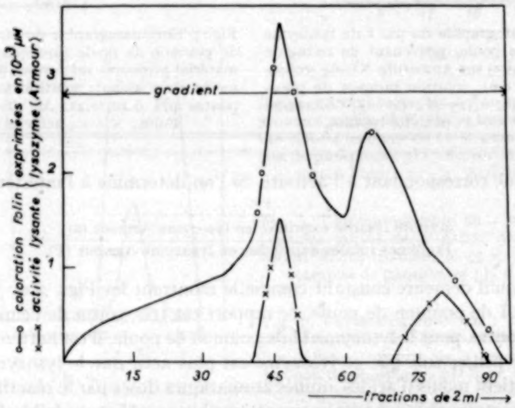

Fig. 1. Chromatographie de 1 mg de matériel primaire de poumon de poule sur Amberlite XE-64 (colonne de 15 × 1 cm), solution tampon de phosphates pH 6.40, et molarité croissant de 0.2 *M* à 0.6 *M*.

Chaque fraction a pu être obtenue à l'état chromatographiquement pur. La fraction I est rechromatographiée sur Amberlite XE-64 avec une solution tampon de phosphates 0.325 *M* à pH 6.40, la résine ayant été équilibrée préalablement avec cette même solution tampon (Fig. 2). La fraction II est rechromatographiée en utilisant une solution tampon de phosphates 0.412 *M* à pH 6.40 (Fig. 3).

Dans les deux cas, une certaine quantité d'impuretés protéiques inactives passent dès le début sans être retenues par la colonne. Après dialyse et lyophilisation, on obtient le lysozyme I et le lysozyme II de poumon de poule. L'activité lysante se retrouve pour chacun des lysozymes entièrement dans un seul pic. La rechromatographie dans les mêmes conditions du matériel ainsi purifié donne naissance à un pic symétrique correspondant à l'activité. Si l'on détermine à l'intérieur de ces pics, le rapport:

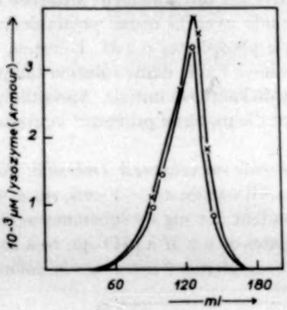

Fig. 2. Chromatographie du pic I de lysozyme de poumon de poule (provenant de 10 mg de matériel primaire) sur Amberlite XE-64 (colonne de 15 × 1 cm); solution tampon de phosphates pH 6.40, 0.325 *M*. O—O, Coloration Folin; ×—×, activité lysante.

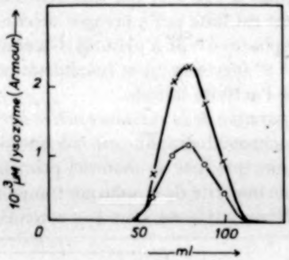

Fig. 3. Chromatographie du pic II de lysozyme de poumon de poule (provenant de 10 mg de matériel primaire) sur Amberlite XE-64 (colonne de 15 × 1 cm); solution tampon de phosphates pH 6.40, 0.412 *M*. O—O, Coloration Folin; ×—×, activité lysante.

$$\frac{\text{activité lysante exprimée en lysozyme Armour (a)}}{\text{Protéines totales exprimées en lysozyme Armour (F)}}$$

on constate qu'il demeure constant comme le montrent les Figs. 2 et 3. Dans le cas du lysozyme I de poumon de poule, ce rapport est très voisin de celui du lysozyme d'oeuf; par contre, pour le lysozyme II de poumon de poule, il est nettement supérieur à 1. On peut penser soit que ce lysozyme est plus actif que le lysozyme d'oeuf (a), soit qu'il contient moins d'acides aminés aromatiques dosés par le réactif de Folin (F).

La chromatographie est extrêmement sensible au pH et un faible écart (± 0.10) provoque soit une adsorption définitive du produit sur la résine, soit un passage sans rétention.

Lysozyme de Nephthys hombergi

Préparation du matériel primaire: 1 kg d'animaux conservés dans l'acétone, puis séchés, contient de 15 à 50 mg de lysozyme. Le matériel primaire est obtenu suivant la même technique que pour le lysozyme de poumon de poule. Il est cependant indispensable pour conserver active la substance lysante de soumettre les extraits aqueux à un chauffage de 3 min à 100° et à pH 4.5. Par ce chauffage, l'activité lysante diminue d'environ 30%, par contre les substances responsables de la désactivation du lysozyme sont totalement détruites.

Séparation de la substance active par chromatographie sur colonnes d'Amberlite: Pour obtenir le lysozyme de *Nephthys hombergi* à l'état chromatographiquement pur, le matériel primaire préalablement purifié sur Amberlite XE-64 est rechromatographié sur une colonne de la même résine en utilisant une solution tampon de phosphates 0.2 *M* à pH 6.60 (Fig. 4). 90% de l'activité lysante se retrouvent dans un seul pic.

On a vérifié que le rapport a/F (voir plus haut) est constant à l'intérieur de ce pic (Fig. 4). Ce rapport est nettement plus grand que dans le cas du lysozyme d'oeuf de poule, ce qui semble en accord avec le nombre peu élevé de résidus de tryptophane par molécule (voir Tableau II). Ici encore la chromatographie est très sensible au pH, comme le montrent les Figs. 5 et 6.

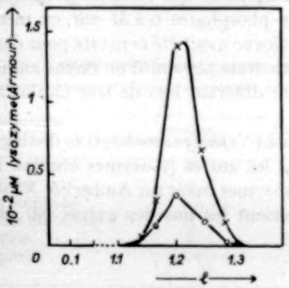

Fig. 4.

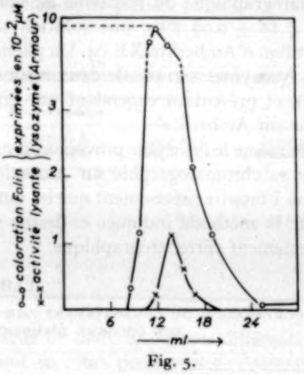

Fig. 5.

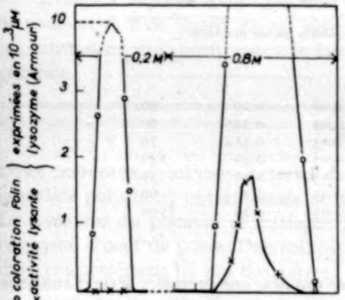

Fig. 6.

Fig. 4. Chromatographie de 10 mg de matériel primaire purifié de *Nephthys hombergi* sur Amberlite XE-64 (colonne de 15 × 4 cm) solution tampon de phosphates pH 6.60, 0.2 *M*. O—O, Coloration Folin; ×—×, Activité lysante.

Fig. 5. Chromatographie de 1 mg de matériel primaire purifié de *Nephthys hombergi* sur Amberleit XE-64 (colonne de 15 × 1 cm); solution tampon de phosphates pH 6.80; 0.2 *M*.

Fig. 6. Chromatographie de 1 mg de matériel primaire purifié de *Nephthys hombergi* sur Amberlite XE-64 (colonne de 15 × 1 cm); solution tampon de phosphates pH 6.50; 0.2 *M* et 0.8 *M*.

DISCUSSION

Le qualificatif de lysozyme[3,6] qui a été donné aux nouvelles substances lysantes décrites ci-dessus se justifie non seulement par le fait que ces substances sont détectées par leur action lysante sur des suspensions de *Micrococcus lysodeikticus*, mais encore par le fait que ces substances sont stables en milieu acide et labiles en milieu alcalin. L'activité est conservée après un chauffage de 3 min à 100° à pH 4.5 alors qu'à pH 8, dans une solution tampon de phosphates 0.2 *M*, l'activité décroît en fonction du temps à température ordinaire et disparaît immédiatement à 100°. Comme tous les autres lysozymes, les trois nouveaux enzymes lysent également des suspensions de *Bacillus megaterium*.

Le lysozyme du poumon de poule se distingue nettement par son comportement chromatographique du lysozyme de blanc d'oeuf de poule qui se chromatographie à pH 7.18 ± 0.08 avec une solution tampon de phosphates 0.2 *M* sur ce même échantillon d'Amberlite XE-64. Un phénomène analogue avait été constaté pour deux autres lysozymes extraits de deux organes différents (rate et rein) d'un même animal (chien), et présentant cependant un comportement différent lors de leur chromatographie sur Amberlite[5].

De même le lysozyme provenant d'un invertébré (*Nephthys hombergi*) se distingue lors de sa chromatographie sur Amberlite de tous les autres lysozymes étudiés. Le Tableau I montre précisément que les différents lysozymes isolés sur Amberlite XE-64 suivant la méthode indiquée ci-dessus se différencient les uns des autres par leur comportement chromatographique.

TABLEAU I

COMPARAISON DU COMPORTEMENT CHROMATOGRAPHIQUE DE QUELQUES LYSOZYMES SUR COLONNE D'AMBERLITE XE-64 DE 15 × 1 cm

Lysozyme	*Solution tampon phosphates*		
	pH	*M*	*ml de sortie*
Oeuf de poule A	7.18 ± 0.08	0.20	90
Poumon de poule I	6.40 ± 0.03	0.325	70
Poumon de poule II	6.40 ± 0.03	0.412	70
Rate de chien	6.50 ± 0.03	0.20	120
Rate de lapin I	6.55 ± 0.03	0.20	20
Rate de lapin II	6.55 ± 0.03	0.20	60
Nephthys hombergi	6.60 ± 0.03	0.20	1100

En ce qui concerne la composition en acides aminés, une première série d'analyses effectuées suivant la méthode de MOORE ET STEIN[6] a montré que le lysozyme du poumon de poule I se distingue nettement du lysozyme d'oeuf de poule. Pour un poids moléculaire[*] voisin de celui du lysozyme d'oeuf de poule, le lysozyme de poumon I contient par exemple 3 fois plus de proline et 2 fois moins d'arginine que celui d'oeuf de poule (Tableau II).

Le lysozyme de *Nephthys hombergi* pour un poids moléculaire analogue[*] se différencie également des autres enzymes lysants par sa composition en acides aminés, comme il ressort d'une première série d'analyses. Les différences principales, comme l'indique le Tableau II, concernent la proline, le tryptophane, la lysine et l'arginine.

De toutes ces observations on peut tirer les conclusions suivantes:

— les lysozymes extraits d'espèces animales différentes (poule, chien[1], lapin[4], *Nephthys hombergi*) bien qu'ayant qualitativement la même activité biologique ont des compositions en acides aminés différentes tout en étant constitués par les mêmes acides aminés. Si certains groupes d'acides aminés ont des teneurs à peu près constantes (Gly + Ala; Asp; Ser + Thr), des différences nettes apparaissent surtout en ce qui concerne la proline, le tryptophane, l'acide glutamique et l'arginine.

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[*] D'après la composition en acides aminés, ce poids moléculaire doit être un multiple d'environ 15,000; la facilité avec laquelle la protéine dialyse à travers certaines membranes de cellophane semble d'autre part indiquer que son poids moléculaire réel ne dépasse pas 15,000.

— la teneur en acides aminés basiques de tous les lysozymes étudiés est grande.

— les lysozymes contenus dans deux organes différents d'un même animal ne sont pas identiques (rate et rein de chien; oeuf et poumon de poule).

TABLEAU II

DIFFÉRENCES MARQUANTES CONCERNANT LA COMPOSITION DE 5 LYSOZYMES

Acide aminé	*Résidus/molécule*				
	Lysozyme d'oeuf de poule	*Lysozyme de poumon de poule*	*Lysozyme de rate de lapin*[4]	*Lysozyme de rate de chien*[1]	*Lysozyme de Nephthys hombergi*
Proline	2	6	5	8	12
Tryptophane	5–6		2	6	1–2
Acide glutamique	5		11	10	8
Lysine	6		6	8	12–13
Arginine	10–11	5–6	6	8	6

L'étude comparée de la structure chimique de deux lysozymes différents (oeuf de poule et rate de chien) qui est actuellement en cours permettra de déterminer si ces substances, qui constituent une famille d'enzymes ont en commun une structure générale.

RÉSUMÉ

Deux nouveaux lysozymes extraits du poumon de poule et de *Nephthys hombergi* (annélide polychète) ont été isolés et obtenus à l'état chromatographiquement pur. Le lysozyme du poumon se distingue par son comportement et sa composition du lysozyme d'oeuf de poule. Une comparaison entre tous ces lysozymes montre qu'ils diffèrent nettement les uns des autres.

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PURIFICATION AND PROPERTIES OF BOVINE ERYTHROCYTE CARBONIC ANHYDRASE

SVEN LINDSKOG

Institute of Biochemistry, University of Uppsala (Sweden)

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SUMMARY

1. A new method for the purification of bovine erythrocyte carbonic anhydrase is described. The procedure involves chromatography on DEAE cellulose ion exchanger and zone electrophoresis in cellulose columns. Two electrophoretic components, having the same specific activities and sedimentation constants, have been discovered.

2. The purified enzyme shows a high degree of homogeneity in electrophoresis and in the ultracentrifuge. The sedimentation constant and molecular weight are 3.06 S and 31,000, respectively.

3. The enzyme contains 16.1 % *N*, 0.34 % S and 0.21 % Zn. The sulfur and zinc contents correspond to 3 and 1 atoms, respectively, per enzyme molecule.

4. The relative merits of different carbonic anhydrase preparations and the possible origin of the two forms of the enzyme are discussed.

INTRODUCTION

Several methods for the purification of carbonic anhydrase have been described in the literature[1]. Most of these methods are based on the use of classical procedures, such as precipitations with salts or organic solvents and treatment with gel adsorbents. Scott and Fisher[2] have reported crystallization of the enzyme but doubts as to the nature of their crystals have been expressed[3]. In addition, the homogeneity of the preparations of Scott and Fisher has been studied in electrophoresis and in the ultracentrifuge[4], and the data indicate the presence of 10–15 % impurities. The purification method most often used is perhaps Method II of Keilin and Mann[5], who found that the enzyme contains firmly bound zinc, which could not be removed without destroying the enzyme activity. Their best preparations appeared to be electrophoretically homogeneous, but the values for the zinc content and specific activity differed from those determined by Scott and Fisher[2].

Since it thus appeared that carbonic anhydrase had not been obtained in a satisfactorily homogeneous state and the studies of its physical properties and chemical composition had given divergent results, attempts have been made to develop a new, simple purification method based on the use of modern separation techniques. The new method retains the ethanol-chloroform treatment of the lyzed

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The following abbreviations are used: DEAE = diethylaminoethyl; Tris = tris(hydroxymethyl)aminomethane.

red cells (see, for example, ref.[6]) but the enzyme solution thus obtained is chromatographed on DEAE cellulose, the final purification being achieved by zone electrophoresis in a vertical column packed with cellulose powder. The purified enzyme, which is obtained in a fairly high yield, appears homogeneous in electrophoresis and ultracentrifuge experiments. The zone electrophoresis experiments, however, revealed the existence of two forms of carbonic anhydrase, differing in mobility but having the same activity and molecular weight. Such an occurrence of several active forms of an enzyme has also been observed in many other cases (see ref.[7]).

EXPERIMENTAL

Assay method

Owing to the great number of activity measurements involved in the chromatographic and electrophoretic experiments, it was considered essential to use a rapid and simple assay method. For this purpose a simplified version of the spectrophotometric method described by CLARK AND PERRIN[8] was found suitable.

Two ml of a 0.05 M Veronal buffer at pH 8.6 containing phenol red to give an extinction at 560 mμ of 1.0, was pipetted into a 1-cm Beckman cuvette. Enzyme (usually 5 μl) was added with a constriction pipet and the cuvette was placed in the cuvette housing of a Beckman DU spectrophotometer equipped with an energy recording adapter. The spectrophotometer was also provided with thermospacers, through which ice water was circulated. When temperature equilibrium was reached (0°–1°), 1 ml of a saturated solution of CO_2 in water (0°) was rapidly added with a syringe. The change of extinction at 560 mμ was followed on a 10 mV Leeds and Northrup recorder, with a chart speed of 0.5 in/10 sec and a response time of 1 sec for full scale deflections.

The activity is expressed as the first order rate constant measured in the presence of enzyme minus the rate constant of the spontaneous reaction. A calibration curve was made in the following way: The extinctions at 560 mμ (D_{560}) were measured for 0.033 M Veronal buffers in the pH range 7.0–8.6, containing an amount of phenol red equal to that used in the activity measurements. From an equation given by CLARK AND PERRIN[8], describing the variation of the CO_2 concentration during the activity measurement as a function of pH and buffer concentration, a curve giving D_{560} as a function of ln $[CO_2]$ could be constructed. By means of this, a curve of ln $[CO_2]$ *vs.* time could be made for each activity measurement, the first order rate constant being calculated as the slope of the initial, linear part of the latter curve. The specific activity is expressed as the activity (sec^{-1}) divided by the protein concentration (expressed as the optical density at 280 mμ) in the cuvette. The latter was calculated from an independent measurement in a 1-cm cuvette after suitable dilution of the original enzyme sample.

Purification

The ***starting material*** was ox blood obtained from the slaughter house. No particular difference in carbonic anhydrase activity could be discovered between fresh blood, cooled in ice immediately after killing the animal, and blood which had been defibrinated by stirring and then stored one day in a cold room.

The *initial purification* follows mainly Steps 1–2 of Method II of KEILIN AND MANN[5] and was performed in the cold room (+ 4°).

Step 1. The red cells were separated from the plasma by centrifuging for 20 min at 2500 × *g*, and then washed three times with an equal volume of 0.9 % NaCl solution.

Step 2. Hemolysis was achieved by adding half a volume of distilled water to the washed erythrocytes.

Step 3. To each liter of lysate, 800 ml of 40 % ethanol and 400 ml of chloroform were rapidly added. The sludge was vigorously stirred for 3 min and then left in the cold for 30 min before centrifuging (15 min at 2500 × *g*). The slightly colored upper phase was finally dialyzed for 40 h against several changes of distilled water.

Ion exchange column chromatography. An almost pure enzyme was obtained after chromatography on a DEAE cellulose column[9]. Since carbonic anhydrase was the first of the adsorbed components to appear during elution, this step could be performed in a very simple way.

The column (dimensions: 6 cm dia., 40 cm length, packed with DEAE cellulose purchased from Distillation Products Industries, Rochester 3, N.Y., U.S.A.), was placed in the cold room and equilibrated with 0.01 *M* Tris–HCl buffer, pH 8.0. To the enzyme solution from Step 3 (4 liters obtained from 4 liters of blood), Tris–HCl buffer, pH 8.0, was added to give a final Tris concentration of 0.01 *M*. The enzyme solution was then filtered through the column at a flow rate of 10–15 ml per min. About 95 % of the protein material, as estimated from the optical density at 280 mμ, was adsorbed by the column, the colored material remaining as a sharp band at the top. The unadsorbed material contained less than 10 % of the total activity and was not further purified.

By a change in the buffer concentration to 0.08 *M*, practically all carbonic anhydrase activity was eluted. The activity separated partially into two peaks

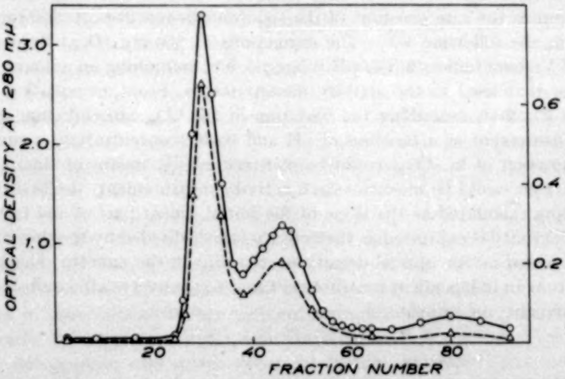

Fig. 1. Chromatography of 4 l of solution from Step 3 on DEAE cellulose. Column dimensions: 6 cm dia., 40 cm length. Material eluted by 0.08 *M* Tris–HCl buffer, pH 8.0. Fraction volume: approx. 30 ml. Fractions number 26–35 (Fraction *b*) and 36–52 (Fraction *a*), respectively, were pooled and purified further by zone electrophoresis. In this figure, as well as in Figs. 2 and 3, circles refer to optical density at 280 mμ and triangles to activity.

(Fractions *b* and *a*), of which the last eluted (Fraction *a*) had a faint yellowish-brown color (Fig. 1). Finally, a very small, colored peak emerged, which contained a small amount of activity. The rest of the proteins could be eluted with 0.05 *M* Tris buffer, pH 8.0, containing 1 *M* NaCl. This fraction had almost no activity. The active fractions (*a* and *b*) were dialyzed against distilled water and freeze-dried. The overall recovery of activity from the DEAE cellulose column was close to 100 %, with 70–80 % in fractions *a* and *b*, while the purification was approximately six-fold.

Zone electrophoresis. The final purification was achieved by zone electrophoresis in a vertical column of the type described by PORATH[10]. The supporting medium was ethanolyzed cellulose powder (obtained from Grycksbo Pappersbruk, Grycksbo, Sweden). The packing and the following operations of the column (dimensions: 2 cm dia., 150 cm length) were performed according to PORATH[10].

The sample was dissolved in 2–4 ml of buffer (phosphate–borate buffer of the following composition: 2.40 g $NaH_2PO_4 \cdot 2H_2O$ and 4.39 g $Na_2B_4O_7 \cdot 10H_2O$ per liter, pH 8.2, $\mu = 0.05$) and dialyzed overnight against 1 l of the same buffer. It was then applied to the top of the column and washed into the column with 10–15 ml of buffer. The electrophoresis was stopped after 90–110 h at 60 mA and 1500 V. The material was displaced with fresh buffer flowing through the column at a rate of about 1 ml/min, and fractions of 4 ml were collected.

To keep pH constant, a membrane pump circulated the buffer between the electrode vessels, which were connected by a siphon. The pumping rate was adjusted so that the buffer level in the cathode vessel was a few cm above that in the anode vessel in order to prevent the electroosmotic flow from moving the slowest component towards the cathode and out of the column.

Fig. 2 shows the elution diagram of an experiment with Fraction *a* from the DEAE cellulose chromatography. Three peaks appeared, the two slowest ones with high activities representing pure carbonic anhydrase A and B, respectively. (See

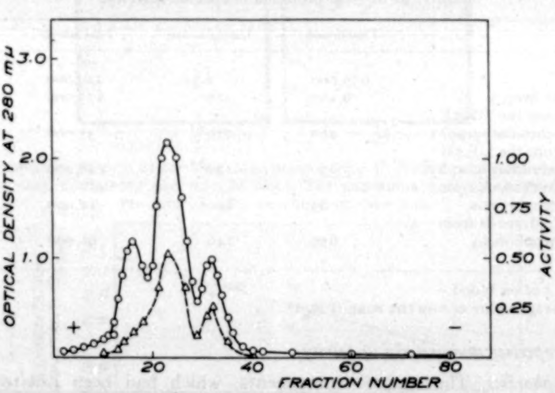

Fig. 2. Zone electrophoresis of Fraction *a*. Column dimensions: 2 cm dia., 150 cm length; buffer: phosphate–borate buffer, pH 8.2, $\mu = 0.05$; current: 60 mA; voltage: 1500 V; time: 90 h; fraction volume: approx. 5 ml. Fractions number 21–27, (carbonic anhydrase A), and 31–35, (carbonic anhydrase B), respectively, were pooled. In this figure, as well as in Fig. 3, the (+) and (—) at the bottom of the diagram indicate the position of the anode and cathode, respectively.

Criteria of Purity.) Fraction *b* (see Fig. 1) gave essentially a single peak (Fig. 3), which was shown by paper electrophoresis to be identical with the slowest component seen in Fig. 2 (carbonic anhydrase B). In Table I, the activity and protein yields in the various steps of the entire preparation procedure are summarized.

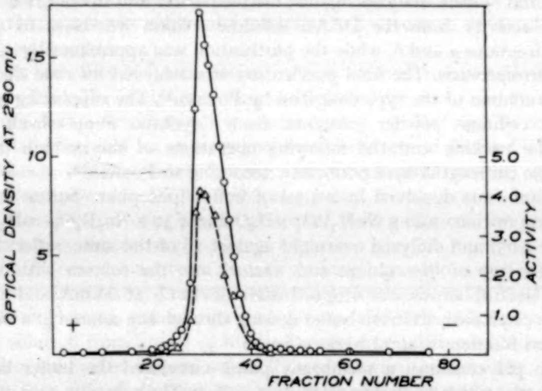

Fig. 3. Zone electrophoresis of Fraction *b*. Column dimensions, buffer, current and voltage as in Fig. 2. Time: 94 h; fraction volume: approx. 4 ml. Tubes number 28–38 were pooled.

TABLE I

PURIFICATION OF BOVINE CARBONIC ANHYDRASE

Total protein is given as the product of the volumes (ml) of the pooled fractions and the absorption at 280 mμ. Specific activity is expressed as defined under *Assay method*. Total activity is the product of total protein and specific activity.

	Total protein	*Specific activity*	*Total activity*	*Yield %*
Hemolysate*	650,000	0.34	220,000	100
Solution from Step 3	9,200	19	175,000	80
Fraction *a* from the DEAE cellulose chromatography	280	110**	31,000	63
Fraction *b* from the DEAE cellulose chromatography	830	130**	108,000	
Carbonic anhydrase A from zone electrophoresis	85	140	12,000	47
Carbonic anhydrase B from zone electrophoresis	650	140	91,000	

* From 4 l of ox blood.
** The average value across the zone (Fig. 1).

Physical properties and criteria of purity

Electrophoresis. The enzyme components, which had been isolated by zone electrophoresis at pH 8.2 were studied in the Tiselius apparatus (Model LKB) at pH 7.0 (phosphate buffer, $\mu = 0.10$) and $+ 0.5°$. The mobilities were calculated as the average of the values for both limbs. Preparations of carbonic anhydrase A and B gave single symmetrical peaks (Fig. 4) with mobilities at this pH and ionic strength of $-2.8 \cdot 10^{-5}$ and $-2.3 \cdot 10^{-5}$ cm²sec⁻¹volt⁻¹, respectively, the latter being in fair agreement with the values found by KEILIN AND MANN[5] and PETERMAN AND HAKALA[4].

Sedimentation studies. All ultracentrifuge experiments were performed with a Spinco Model E Ultracentrifuge at 59,780 rev./min and 20.0°. Before a run, the sample was dialyzed overnight against the buffer (the phosphate–borate buffer, pH 8.2, used in the zone electrophoresis experiments containing also 0.10 *M* NaCl).

The sedimentation constants were calculated and corrected to give the values in water at 20.0° ($s_{20,w}$) according to SVEDBERG[11].

Preparations of carbonic anhydrase A and B both gave symmetrical peaks

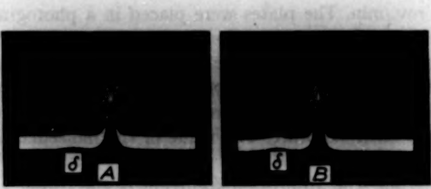

Fig. 4. Free electrophoresis in phosphate buffer, pH 7.0, $\mu = 0.10$, of carbonic anhydrase A and B. The photographs are of the ascending boundaries. The potential gradient was 6.4 V/cm. The peak marked δ is a salt gradient. The exposures were taken 168 min (A) and 187 min (B), respectively, after the start.

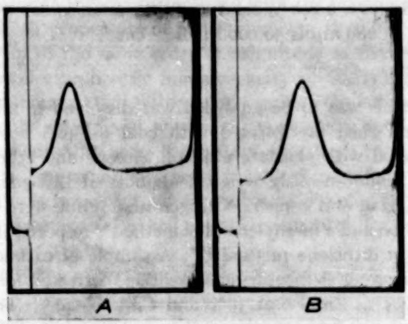

Fig. 5. Sedimentation pattern of carbonic anhydrase A and B. Buffer: phosphate-borate buffer, pH 8.2, $\mu = 0.05$, containing also 0.10 *M* NaCl. The exposures were taken 74 min (A) and 119 min (B) after reaching 59,780 r.p.m.

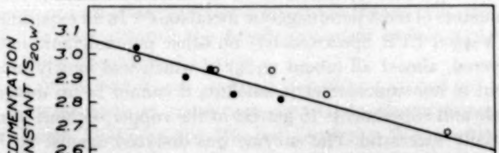

Fig. 6. The sedimentation constant of carbonic anhydrase as a function of the protein concentration. Solid circles refer to carbonic anhydrase A, while open circles refer to carbonic anhydrase B. The straight line was drawn with the aid of the least-square method.

(Fig. 5) with the same extrapolated sedimentation constants. The value of $s_{20,w}$, extrapolated to zero protein concentration, is 3.06 S (Fig. 6).

Molecular weight. The partial specific volume, $\bar{V}$, was determined for a carbonic anhydrase B solution, which had been extensively dialyzed against distilled water. The determination[12] was performed with a 5 ml pycnometer at 25° and with a protein concentration of 56.7 mg/ml. The value thus obtained was $\bar{V} = 0.742$ ml/g.

The molecular weight was determined from measurements during the approach to sedimentation equilibrium as described in ref.[12]. The apparatus, buffer and temperature were the same as in the sedimentation studies and the ultracentrifuge was operated at 9,945 rev./min. The plates were placed in a photographic enlarger and the ultracentrifuge patterns were traced onto graph paper. To facilitate the calculations the enlarger was adjusted so that the final enlarging factor was 10.00. Measurements were made from 9 exposures taken 70 to 200 min after attaining full speed and the molecular weight was computed from the patterns at the meniscus and the bottom of the cell of each exposure. The value of the molecular weight thus found was 31,000 ± 1000.

Ultraviolet spectrum. The absorption spectrum in the region 240–300 mμ of the purified enzyme is typical for a "simple" protein, having a maximum at 280 mμ and a minimum at 251 mμ. The ratio D_{280}/D_{260} is 1.6. The enzyme contains an unusually high amount of aromatic amino acids, giving an optical density at 280 mμ in a 1-cm cell of 1.80 for a 0.100 % solution. With a molecular weight of 31,000, the specific absorbancy at 280 mμ is 56,000 l mole^{-1} cm^{-1}.

Analytical data

The enzyme which was to be analyzed was dissolved in and dialyzed against deionized water, and then precipitated with cold (—40°) absolute ethanol. The precipitate was washed with absolute ethanol, acetone and ether, and air-dried at room temperature. Simultaneously weighed aliquots of this sample were analyzed for nitrogen, sulfur, zinc and copper. Nitrogen and sulfur were determined with a micro-Kjeldahl method and a methylene-blue method*, respectively, while the metals were analyzed with a dithizone method[13]**. A sample of carbonic anhydrase thus prepared had the following elementary composition, corrected for moisture content: N = 16.1 %, S = 0.34 %, Zn = 0.21(3) % and Cu = 0.049 %. The sulfur, zinc and copper contents correspond to 3.3, 1.0 and 0.24 atoms, respectively, per enzyme molecule. The enzyme was not analyzed for other metals, but electron-spin-resonance measurements were performed, since such measurements should reveal the presence of significant amounts of most paramagnetic metal ions[14]. In an experiment (performed with a Varian V-4500 EPR Spectrometer) no other paramagnetic ions than copper could be discovered, almost all (about 99 %) of which was ionicly bound. Since the copper is present in non-stoichiometric amounts it cannot be an integral part of the enzyme molecule and experiments to get rid of the copper without affecting the zinc have been partially successful. The enzyme was dialyzed against 10^{-3} *M* *o*-phenanthroline in water (pH about 7), and then dialyzed against deionized water before the precipitation with ethanol. The copper content was now reduced to 0.08 atoms of copper per enzyme molecule, while the zinc content had not been significantly changed.

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* L. GUSTAFSSON, to be published.

** The analyses were performed by Dr. A. BENGTSSON and Dr. LILLY GUSTAFSSON at the Department of Analytical Chemistry, University of Uppsala.

NOTE ADDED IN PROOF

Further electron-spin resonance studies have revealed that the peak assigned to ionic Cu^{2+} might also be due to Fe^{3+}. However, since this peak completely disappears after the dialysis against *o*-phenanthroline, the only paramagnetic contaminant remaining is a small amount of covalently bound copper, found also by the chemical analysis.

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DISCUSSION

The new purification method described in this paper appears to be a definite improvement over the methods described earlier[1]. Though the yield of purified enzyme is about the same as that obtained by, for example, KEILIN AND MANN[5], the new procedure is simpler, giving a well-defined end product, homogeneous in electrophoretic and sedimentation experiments. These data, together with the zinc content, are very similar to those for the enzyme prepared by the method of SCOTT[2, 4]. A few experiments to determine the specific activity with the manometric method, though not performed with all proper precautions[15], indicate that the present preparation has a specific activity of the same order of magnitude as that found by SCOTT[2]. It is possible that the electrophoretic inhomogeneity of SCOTT's preparation[4] can be explained by the existence of two active components of the enzyme, but the sedimentation behavior, on the other hand, shows inhomogeneities not found here.

From the zinc content, which, though differing from the data of KEILIN AND MANN[5] (0.33 %), is in agreement with those of SCOTT[2] (0.20–0.23 %), it can obviously be concluded that the enzyme contains *one* zinc ion per molecule. This zinc ion is very firmly bound, since it remains attached to the protein after dialysis against *o*-phenanthroline.

In the particular preparation described under *Purification*, carbonic anhydrase B was the dominating form of the enzyme. In about one preparation out of three, however, the A component existed in larger amounts than the B component, and the relative amounts of the two forms varied from one preparation to another. Experiments to prepare the enzyme with omission of the ethanol–chloroform step indicate that only one form of carbonic anhydrase is present in the red cells, the electrophoretic difference probably arising during the drastic treatment in Step 3 of the initial purification. This is being investigated further.

Preparations of purified *human* carbonic anhydrase, carried out at this institute by Mr. P.-O. NYMAN, have in some cases given two electrophoretically different, equally active forms, but here too the polydispersity is probably a preparation artifact. The enzyme from human erythrocytes, though having the same sedimentation constant, shows many differences compared with the bovine enzyme, and parallel studies of the physical and chemical properties, including the nature of the metal binding sites, are being planned.

ACKNOWLEDGEMENTS

The author wishes to thank Professor A. TISELIUS for his kind interest. To his teacher, Dr. BO G. MALMSTRÖM, the author wishes to express his deep gratitude for suggesting this research and for continual discussions during its performance. Valuable technical assistance by Mrs. INGA EKSTRAND is also acknowledged. This investigation has been supported by the Swedish Natural Science Research Council.

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ACID DESOXYRIBONUCLEASE ASSAY BY THE METHYL GREEN METHOD

N. B. KURNICK AND GEORGIANNA SANDEEN

Veterans Administration Hospital, Long Beach, Calif. and Department of Medicine,
University of California Medical Center, Los Angeles, Calif. (*U.S.A.*)

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SUMMARY

The methyl green method of DNase assay is applicable in the acid range as well as at pH 7.5. As compared to the DNA degradation product assay, it has the advantages of greater technical simplicity, sensitivity, versatility, and economy of materials. It measures directly the enzymic degradation of DNA and is independent of secondary enzyme effects, such as of nucleotidases and phosphatases, which may influence the rate of formation of acid soluble products. Advantages of the methyl green method compared to other assay methods have been previously discussed[1].

Abbreviations: DNA, deoxyribonucleic acid; MG, methyl green; DNase, deoxyribonuclease.

INTRODUCTION

The methyl green method for assay of alkaline desoxyribonuclease (DNase) described in 1950[1,2] is based on the progressive reduction of methyl green affinity for desoxyribonucleic acid (DNA) as the molecular configuration is altered by depolymerization. As enzymic depolymerization of the DNA-methyl green complex progresses, methyl green is liberated and fades at neutral pH, lowering the O.D. of the substrate solution. The original publication[1] of the method for determining DNase activity at pH 7.5 included a brief description for assay at pH 5.0. For the latter, post-reaction adjustment of the solution to pH 7.5 is performed to allow the liberated methyl green to fade. Since KOWLESSAR *et al.*[3] have stated that the methyl green method is not suitable for acid DNase assay, and since we have substituted $MgCl_2$ for $MgSO_4$ for reasons discussed elsewhere[4], we believe a detailed re-statement of the acid DNase assay may be appropriate. Some observations on the comparison of this method to one based on determination of acid soluble DNA degradation products are also presented. We have used our method successfully for the assay of acid DNase in a variety of tissues[5-7].

EXPERIMENTAL

The substrate mixture consists of: 100 ml 2 mg/ml aqueous DNA*; 100 ml 0.04 % aqueous methyl green**; 200 ml 0.05 *N* acetate buffer, pH 4.6; 100 ml 0.6 *N* magnesium chloride; 700 ml distilled water and 1 ml 1 % aqueous sodium ethyl mercurithiosalicylate ("Merthiolate")***.

The mixture is stable in the refrigerator for several weeks. The pH should be 4.6 after the addition of the enzyme solution and at the end of the reaction period. The pH and concentration of magnesium chloride were selected on the basis of pH and electrolyte concentration optima studies[4].

To 12 ml of substrate mixture, 1 ml of tissue homogenate is added. Immediately, and at 5 appropriate intervals of incubation at 37°, 2 ml-aliquots are pipetted into 2 ml of a mixture of one part 0.33 *M* sodium citrate and three parts 0.05 *M* tris (hydroxymethyl)aminomethane (Tris) buffer[11], pH 7.5. This reagent stops the reaction by increasing salt concentration and by adjusting the pH to 7.5, at which pH the uncombined methyl green fades[12]. The sample tubes are covered with Parafilm, mixed, and allowed to stand in subdued light at room temperature overnight to assure complete fading of free methyl green. The O.D. is then read at 640 mμ. The slope of the curve of the O.D. *versus* reaction time determines the rate of enzyme activity. The unit of enzyme activity, as previously defined[1], is that which causes a reduction in O.D. of 1.0 per min/ml reaction mixture. Thus, if a 1:200 spleen homogenate were found to cause a reduction in O.D. (ΔE) of 0.240 in 240 min, the calculations would be:

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* Calf thymus sodium desoxyribonucleate prepared by the method of MIRSKY AND POLLISTER[8]. This material is available from Worthington Laboratories, Freehold, New Jersey. DNA prepared by the method of SIMMONS *et al.*[9,10] has also been successfully used. Most commercial preparations are degraded and are not suitable. The stock solution is stable for weeks in the refrigerator, without change in viscosity.

** The stock methyl green (National Aniline ethylated hexamethyl pararosanaline C.I. No. 685, cert. No. NG-26) solution is 0.4 % in water, which has been exhaustively extracted with chloroform.

*** "Merthiolate" is the trademark of Eli Lilly and Company for Thimerosal, made by it.

$$\frac{0.240\ (\Delta E)}{240\ (\text{min})} \times \frac{13\ (\text{ml reaction mixture})}{0.005\ (\text{g spleen in mixture})} \times \frac{4\ (\text{ml final solution})}{2\ (\text{ml reaction aliquot})} = 5.2\ \text{units/gram spleen}$$

In our analyses of mouse tissues we have found that a reaction time of 4 h allows very high dilutions of tissue to be used (*e.g.*, spleen 1:200–1:400 wet weight to volume). This not only permits small organs such as mouse thymus[5–7], to be tested individually, but also minimizes precipitation of proteins with the substrate at acid pH.

Occasionally tissue homogenates must be used at concentrations which cause precipitation on addition to the substrate. The precipitate may be a fine, easily dispersed, colorless one which can be pipetted uniformly and does not require any modification of the test procedure. Sometimes a deep green, fibrous mass of protein-DNA-methyl green forms, which cannot be pipetted. If it is not permissible to work with a much lower concentration of enzyme solution to avoid precipitation, the test may be set up in individual tubes for each time interval. Each tube contains 1.9 ml substrate and 0.1 ml homogenate*. At appropriate intervals, 2 ml Tris–citrate mixture is added. The precipitate usually dissolves at pH 7.5. If an uncolored sediment remains, it may be removed by centrifugation, but a green sediment makes the test unreliable. Precipitated methyl green-DNA in the reaction mixture, if completely redissolved at pH 7.5, apparently does not interfere, since the enzyme activity remains proportional to enzyme concentration.

If preparations of very high activity are to be tested, it is permissible to increase the DNA and methyl green concentration and reduce the reaction time to avoid depletion of the substrate before the end of the reaction period. As noted below, a range of DNA concentrations from 0.077 to 0.308 mg/ml (1/2 to 2 × the usual substrate) does not affect the enzyme activity.

For the determination of acid soluble degradation products of DNA, the following modification of the method of ALLFREY AND MIRSKY[13] was used. The substrate consisted of: 4 ml 2 mg/ml aqueous DNA, 1 ml 0.6 N $MgCl_2$ and 7 ml 0.1 N acetate buffer, pH 4.6. These modifications were made on the basis of our observations[4] that, like the methyl green method, this method gives higher activities with the given pH and $MgCl_2$ concentration than with the conditions prescribed by ALLFREY AND MIRSKY. To this substrate, 1 ml of tissue homogenate was added. Immediately and at appropriate intervals of incubation at 37°, 3 ml reaction mixture were withdrawn into 1 ml cold 10% trichloroacetic acid, and centrifuged at 5°. Acid soluble degradation products were determined by the diphenylamine color reaction for desoxyribose[14] using 1.5 ml of the supernatant and 3 ml diphenylamine reagent. The activity was measured from the slope of the curve of the O.D. of the diphenylamine color reaction *versus* time of incubation.

Typical DNase activity curves obtained with the methyl green method are shown in Fig. 1 for three concentrations of enzyme. Fig. 2 shows similar activity curves determined from the acid-soluble degradation product assay. The enzyme sample for these two figures was not the same. Fig. 3 is a graph of enzyme activity against enzyme concentration for each assay method. The same enzyme preparation was used for each assay in this comparison. This figure illustrates that both methods yield enzyme activities proportional to homogenate concentration. Three different

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* These proportions are not exactly the same as the standard method. For comparison, a correction factor of 20/13 must be applied to ΔE.

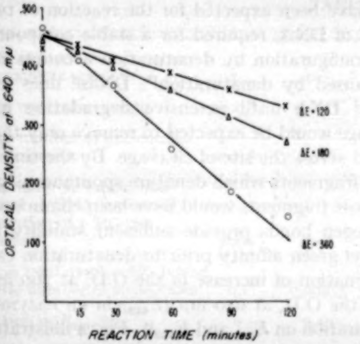

Fig. 1. Acid DNase activity by methyl green assay. Grams of spleen per 13 ml reaction volume: ○, 0.010 g; △, 0.005 g; ×, 0.0033 g. The *ΔE*'s shown on the curve are for 120 min.

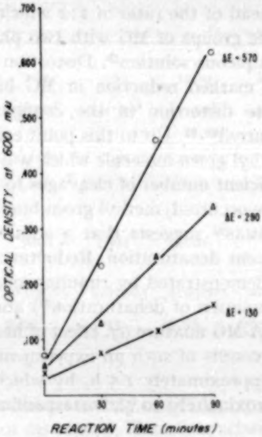

Fig. 2. Acid DNase activity by DNA degradation product assay. Grams of spleen per 13 ml reaction volume: ○, 0.040 g; △, 0.020 g; ×, 0.010 g. The *ΔE*'s shown on the curve are for 90 min.

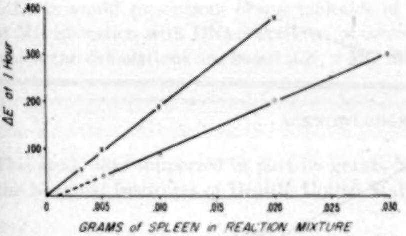

Fig. 3. Change in enzyme activity with change in enzyme concentration. × = methyl green assay, at 4 different concentrations of DNA (see text). ○ = degradation product assay. The same enzyme was used for all the points in Fig. 3.

DNA concentrations (0.154, 0.231, 0.308 mg/ml) were used at each enzyme concentration in the methyl green method. In addition, a substrate containing 0.077 mg/ml DNA was tested at the two lower enzyme concentrations. The change in O.D. (*ΔE*) with the four substrates did not vary more than 0.010 from the mean for each enzyme concentration.

The acid solubility method is approximately one-half as sensitive as the methyl green method and requires four times as much DNA per determination as does the standard methyl green method. Since acid soluble products appear only after very extensive degradation of the DNA[15,16] there is a significant lag period after the enzyme is added. This becomes more marked at low enzyme concentration, so that the curve does not pass through zero as it does for the methyl green method.

DISCUSSION

In previous studies on methyl green (MG) affinity for DNA[17,18] we suggested that the steric configuration of the DNA molecule determines MG binding. The steric factors were considered responsible for the ratio of approx. 1 MG per 10 phosphate groups instead of the ratio of 1:2 which might have been expected for the reaction of two basic groups of MG with two phosphates of DNA, required for a stable compound in aqueous solution[18]. Distortion of the configuration by denaturation accounts for the marked reduction in MG binding caused by denaturation[19]. DNase does not cause distortion in the configuration of DNA until extensive degradation has occurred[20, 21]. Up to this point each cleavage would be expected to remove only that methyl green molecule which was attached across the site of cleavage. By the time a sufficient number of cleavages to produce fragments which denature spontaneously[21] have occurred, methyl green binding to those fragments would have been eliminated. THOMAS[21] suggests that 3 adjacent hydrogen bonds provide sufficient stability to prevent denaturation. Reduction of methyl green affinity prior to denaturation can be demonstrated by simultaneous determination of increase in the O.D. at 260 mμ (a function of denaturation[21]) and fall in the O.D. at 640 mμ (E_{640}) in an enzyme-DNA-MG mixture (*cf.* effect of heat denaturation on E_{260} and E_{640}[1]). Fig. 4 illustrates the results of such an experiment. It demonstrates a lag in denaturation (E_{260} rise) of approximately 1.5 h, by which time the methyl green affinity has been reduced approximately 20 %, corresponding to about 500 cleavages (see Table I).

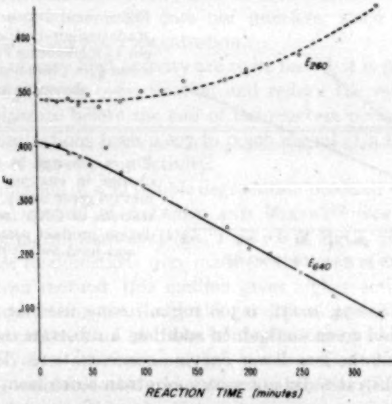

Fig. 4. Simultaneous determination of effect of acid DNase on E_{260} and E_{640} of MG–DNA substrate. 6 ml of the enzyme, 1:400 normal mouse spleen aqueous homogenate supernatant, was added to 72 ml of substrate. The solid line, marked E_{640}, show the fall in E_{640} with time, diluted as in our standard methyl green method. The interrupted line, marked E_{260}, shows the rise in E_{260} with time of the same Tris–citrate diluted enzyme substrate mixture, further diluted 1:2 with Tris–citrate. The blank for the E_{260} readings contained all the constituents of the test sample except DNA.

The calculations for Table I are based upon the ratio of DNA to MG of 10 P per MG for the "native" DNA. It is assumed that one MG is released with each cleavage. From these calculations the findings of ROSENKRANZ AND BENDICH[22] that no significant change in MG binding occurs on depolymerization, without denaturation of DNA, from molecular weight $6.7 \cdot 10^6$ to $1.7 \cdot 10^5$ may be explained: the predicted loss of methyl green binding for this degree of depolymerization is only 2 %. The calculations are consistent with our findings that DNase activity is independent of

TABLE I

Number of fragments (number of complete scissions + 1)	1	10	40	200	500	1000	2000
Average molecular weight	$6 \cdot 10^6$	$6 \cdot 10^5$	$1.5 \cdot 10^5$	$3 \cdot 10^4$	$1.2 \cdot 10^4$	$6 \cdot 10^3$	$3 \cdot 10^3$
Average number of nucleotides per fragment	$2 \cdot 10^4$	$2 \cdot 10^3$	500	100	40	20	10
Total number MG molecules bound to DNA (assuming liberation of 1 MG per scission)	2000	1991	1961	1801	1501	1001	1
MG/P = total number MG bound /total number of nucleotides	0.1	0.09955	0.09805	0.09005	0.07505	0.05005	0.00005
Reduction in E_{640}, %	0	0.45	1.95	9.95	24.95	49.95	99.95

the initial molecular weight of the DNA substrate, so that a variety of DNA preparations may be used for the assay, provided they are not denatured. Thus, four scissions of each of 10 molecules of molecular weight $6 \cdot 10^5$ would produce the same change in O.D. as would 40 scissions of one molecule of molecular weight $6 \cdot 10^6$. The linearity of MG liberation with DNase activity, of course, is inherent in the assumption upon which the calculations are based (*i.e.*, 1 MG liberated per cleavage).

ACKNOWLEDGEMENTS

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AMINO ACID ACTIVATION IN EXTRACTS FROM IMMATURE ERYTHROCYTES OF DUCKS

J. VAN ROOD, J. BOOT, J. W. BRUNING AND A. KASSENAAR
Department of Clinical Endocrinology and Metabolic Diseases, University Hospital, Leiden (The Netherlands)

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SUMMARY

Extracts from immature erythrocytes of the duck catalyse the amino acid-dependent phosphate exchange between ATP and pyrophosphate. At pH 5.15 an active enzyme fraction precipitated. Among the amino acids tested, only leucine, valine, isoleucine and glutamic acid were found to be active. The pH optimum of the exchange was about 5.5 in the presence of added glutamic acid and about 7.3 in the presence of added leucine. No exchange between ATP and orthophosphate was demonstrable.

INTRODUCTION

It is known that the immature reticulocyte, in contrast to the mature erythrocyte, is able to incorporate amino acids into protein. In particular, the incorporation of amino acids into haemoglobin has been studied extensively (see *e.g.* KRUH AND BORSOOK[1], KASSENAAR, MORRELL AND LONDON[2]).

In the present study the exchange of ^{32}P between ATP and P^{32}P, indicating the formation of aminoacyladenylates according to DAVIE, KONINGSBERGER AND LIPMANN[3], HOAGLAND, KELLER AND ZAMECNIK[4] and others, was studied in the immature erythrocyte of the duck.

MATERIAL

Enzyme preparation

In order to increase the number of immature erythrocytes, the ducks were given 20 mg phenylhydrazine–HCl per day by intravenous injection for 3 days. They were bled from the jugular vein on the 5th day. The blood was collected with heparin as anticoagulant. The plasma and white cells were removed by centrifugation and the erythrocytes washed twice with 0.9% saline.

The washed and packed erythrocytes were homogenized in a Bühler-apparatus for 45 sec at full speed and a temperature of + 4°C. The homogenate was centrifuged at 18,000 *g* for 120 min at + 4°C. The clear dark red supernatant obtained will be referred to as the ***crude enzyme preparation.***

This crude preparation was fractionated by adding 0.1 *M* acetic acid. The precipitates successively formed at pH 6.2, 5.8 and 5.15 were isolated by high speed centrifugation. These fractions were redissolved in 0.1 *M* Tris buffer* at pH 7.6 to a final volume identical to the volume of the crude enzyme preparation.

When not immediately tested for ATP–PP exchange capacity the solutions were stored at — 20°C.

Reagents

Radioactive inorganic pyrophosphate ($P^{32}P$) was prepared by high temperature fusion of Na_2HPO_4, labelled with ^{32}P. The preparations were checked for residual orthophosphate and invariably contained less than 2 %.

The ATP was the disodium salt from Sigma. The amino acids were obtained from Hofmann–La Roche (Basel). The concentration of the amino acid mixture was in most experiments 1 mmole of each amino acid per ml distilled water. The composition of the various mixtures used is given in the experimental section.

Assay procedure

The incubation mixtures for the ATP–$P^{32}P$ exchange reaction consisted of either phosphate or Tris-buffer of various pH, 5–10 μmoles $MgSO_4$, 10 μmoles $P^{32}P$, 10 μmoles ATP, 100 μmoles NaF, amino acids and various amounts of extract in a total volume of 0.8–1.2 ml. After shaking at 37° for 20 min, the reaction was terminated by the addition of 2 ml 5 % TCA. The protein precipitated was removed by centrifugation.

The nucleotides were separated from inorganic phosphate and pyrophosphate by the method of CRANE AND LIPMANN[6]**.

Inorganic phosphate was determined by the method of BRIGGS[7]. The ^{32}P-activity was counted in a liquid counter tube, as described by VEALL[8].

RESULTS

The crude enzyme preparation showed a considerable activity in catalysing the exchange reaction between ATP and $P^{32}P$. When no amino acids were added the % exchange, calculated according to DEMOSS AND NOVELLI[5], during 20 min incubation at 37° in phosphate buffer at pH 6.5, amounted to a mean of 46.5 %. When an amino acid mixture, containing valine, histidine, leucine and phenylalanine in a total amount of 10 μmoles was added, this value was increased to about 60 %.

After dialysis of this crude enzyme preparation for 17 h against 0.05 *M* phosphate buffer at pH 6.5 at + 4°C, the % exchange was found to be 18 % when no amino acids were added and 37 % after addition of amino acids.

After heating in a boiling waterbath for 15 min, the activity was negligible.

Only the fraction obtained by precipitation at pH 5.15 was found to be active. The results of a typical experiment are given in Table I. In the further experiments only this fraction was used.

It was found that the ATP–PP exchange was increased by adding leucine, isoleucine, valine or glutamic acid (Table II), while histidine, glycine, tyrosine, aspartic acid, arginine, threonine, lysine, phenylalanine, serine and tryptophan showed no effect.

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* Tris(hydroxymethyl)aminomethane, Sigma.

** The norit A was kindly supplied by the Norit Co., Amsterdam.

TABLE I

AMINO ACID-DEPENDENT ATP–$P^{32}P$ EXCHANGE WITH CRUDE ENZYME PREPARATION AND WITH pH 5.15 PRECIPITABLE FRACTION

The data are expressed as % exchange, calculated according to DEMOSS AND NOVELLI[5]. Conditions: 0.3 ml enzyme preparation, 50 µmoles buffer pH 6.5, 10 µmoles ATP, 10 µmoles $P^{32}P$, 100 µmoles NaF, 5 µmoles $MgSO_4$; amino acid mixture: equimolar amounts of leucine, valine, histidine, phenylalanine, isoleucine, tyrosine, serine, glycine, arginine, glutamic acid, aspartic acid, alanine, lysine, threonine; total volume, 1 ml.

Amino acid (µmoles)	*0*	*30*
Crude enzyme prepn.	18	46
pH 5.15 fraction	6	32

TABLE II

THE ACTIVITY OF INDIVIDUAL AMINO ACIDS IN THE ATP–$P^{32}P$ EXCHANGE REACTION IN THE PRESENCE OF THE pH 5.15 PRECIPITABLE FRACTION FROM DIFFERENT BATCHES OF IMMATURE DUCK ERYTHROCYTES

The data are presented as mentioned in Table I. Conditions: 0.3 ml extract, 50 µmoles buffer pH 6.5, 10 µmoles ATP, 10 µmoles $P^{32}P$, 100 µmoles NaF, 5 µmoles $MgSO_4$.

Amino acid (µmoles)	*0*	*30*	*increase in % exchange*
Glutamic acid	8	21	13
Isoleucine	8	21	13
Valine	3	10	7
Leucine	7	24	17

TABLE III

THE EFFECT OF pH ON THE AMINO ACID-DEPENDENT ATP–$P^{32}P$ EXCHANGE WITH THE pH 5.15 PRECIPITABLE FRACTION

Data expressed in % exchange. Conditions: 0.3 ml extract, 50 µmoles buffer, 10 µmoles ATP 10 µmoles $P^{32}P$, 100 µmoles NaF, 5 µmoles $MgSO_4$, 30 µmoles amino acid (for the mixture composition see Table I); total volume 1.2 ml.

	pH		
	5.6	*6.5*	*7.6*
Without amino acids	6	6	6
Amino acid mixture	49	34	23
Glutamic acid	40	33	21
Leucine	8.5	24	24
Valine	6	14	17
Isoleucine	8	17	17

The results assembled in Table III show that glutamic acid is more effective at a lower pH, while leucine, valine and isoleucine exert their influence at higher pH-values.

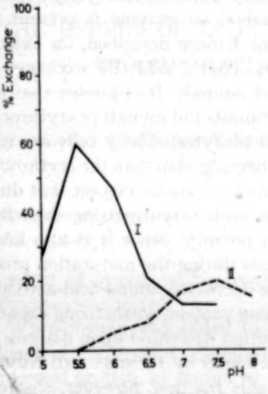

Fig. 1. The effect of the pH of the incubation medium on the amino acid-dependent ATP–PP exchange. Conditions: 0.3 ml enzyme solution (pH 5.15 precipitable fraction) 50 μmoles buffer of different pH, 10 μmoles ATP, 10 μmoles $P^{32}P$, 100 μmoles NaF, 5 μmoles $MgSO_4$, 30 μmoles amino acid, total volume 1.2 ml. I: glutamic acid; II: leucine.

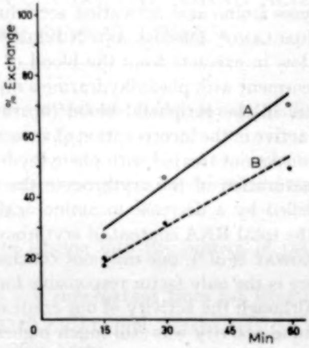

Fig. 2. The effect of incubation time on the amino acid-dependent ATP–PP exchange. Conditions: 0.4 ml enzyme solution (pH 5.15 precipitable fraction from 2 different groups of ducks). Further conditions as indicated under Table I. A: enzyme from ducks A; B: enzyme from ducks B.

TABLE IV

THE EFFECT OF AMINO ACIDS ON THE ATP–P AND ON THE ATP–PP EXCHANGE REACTION WITH THE pH 5.15 PRECIPITABLE ENZYME FRACTION

Conditions: 0.4 ml extract, 30 μmoles buffer, 10 μmoles ATP, 10 μmoles $P^{32}P/^{32}P_i$, 5 μmoles $MgSO_4$, 100 μmoles NaF, amino acids; total volume 1.2 ml.

Amino acid (µmoles)		0	30
		% Exchange	
Pi–ATP exchange	amino acid mixture	2.4	2.5
	leucine	2.0	2.3
	glutamic acid	2.4	1.9
PP-ATP exchange	amino acid mixture	9	33
	leucine	8	18
	glutamic acid	9	57

Fig. 1 demonstrates that the pH optimum of the effect of glutamic acid is about 5.5, and of leucine about 7.3.

Fig. 2 shows that under our conditions the % exchange increases linearly with time during the first hour. Furthermore, it shows that the activities of enzyme preparations from the blood of different ducks varied greatly. Table IV shows that the exchange reaction is specific for pyrophosphate. No exchange between orthophosphate and ATP could be detected.

DISCUSSION

The results presented above suggest that in immature duck erythrocytes, abundant in the blood of ducks treated with phenylhydrazine, an enzyme is present that catalyses amino acid activation according to the mechanism described, for example, by HOAGLAND[4], DEMOSS AND NOVELLI[5] and others. The % ATP–PP exchange was very low in extracts from the blood of non-treated animals. It is known that after pretreatment with phenylhydrazine a much greater number of immature erythrocytes appears in the peripheral blood (up to 100% reticulocytes). These cells are much more active in the incorporation of amino acids into haemoglobin than the erythrocytes from ducks not treated with phenylhydrazine[2]. Our observations suggest that during the maturation of the erythrocyte the decrease in protein-synthetizing capacity is parallelled by a decrease in amino acid-activating potency. Since it is also known that the total RNA content of erythrocytes decreases during the maturation process (HOLLOWAY *et al.*[9]), one may not conclude that the decreased amino acid-activating potency is the only factor responsible for the declining protein-synthetizing capacity.

Although the activity of our crude enzyme solution decreased upon dialysis, the remaining activity was still much higher than that found by DEMOSS AND NOVELLI in dialysed *E. Coli* extracts. The pH 5.15 precipitable fraction, however, showed a similar activity in the absence of added amino acids to that found by these authors.

It was somewhat surprising to find that only a very limited number of amino acids were active. The possibility has to be considered that our experimental conditions were not optimal for activation. For instance, the great difference of the pH optimum for leucine and glutamic acid suggests that at another pH than that used positive effects would have been found with the seemingly inactive amino acids. Furthermore, the concentration of these amino acids may have been too low. It is also possible that during the preparation of the pH 5.15 precipitable fraction some of the amino acid-activating enzymes are destroyed.

ACKNOWLEDGEMENT

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THE EFFECT OF INJECTED INSULIN ON THE AMINO ACID INCORPORATING SYSTEM OF RAT LIVER*

RUTH G. DOELL**

Department of Physiology, Tufts University School of Medicine, Boston, Mass. (U.S.A.)

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SUMMARY

The effect of insulin on the incorporation of leucine into the protein of rat liver microsomes was tested.

When insulin was added to the 15,000 × *g* supernatant from rat liver in the presence of [^{14}C]leucine, no effect was observed in the final specific activity of the microsomal protein, as compared with the control value.

However, when insulin was injected into the animal 12 or 18 h prior to sacrifice, there was an increase of approximately 40 % in the specific activity of the microsomal protein following incubation. Part of the stimulation was shown to reside in the supernatant fraction and part in the microsomes.

INTRODUCTION

Since insulin is known to prevent the nitrogen loss consequent upon the deranged protein metabolism of the diabetic state, it has been assumed to play a role in the normal regulation of protein metabolism. However, it was not until the advent of radioactive tracers that an increased incorporation of amino acids into protein could be demonstrated under the influence of insulin[1-3].

The development of a cell-free system for the study of protein synthesis[4] suggested the possibility of more closely defining a locus of action for this hormone. In the experiments described in this paper we have demonstrated that insulin injected into rats 12 or 18 h prior to sacrifice increases the incorporation of [^{14}C]leucine into the protein of isolated microsomes.

EXPERIMENTAL

Wistar rats maintained on a Purina laboratory chow diet were used in these experiments. 12 or 18 h prior to sacrifice the experimental animals received a subcutaneous injection of 40 units/kg body weight of protamine-zinc insulin (Lilly).

Abbreviations: ATP, adenosine triphosphate; RNA, ribonucleic acid; PCA, perchloric acid; HDP, hexose diphosphate; TCA, trichloroacetic acid.

[*] This work was presented at the meeting of the Federation of American Societies for Experimental Biology, Atlantic City, April, 1959.

[**] Present Address: Department of Radiology Stanford University School of Medicine, Palo Alto, Calif. (U.S.A.).

The amino acid incorporating system was essentially that of ZAMECNIK AND KELLER[4] except that the homogenizing medium contained 0.35 M sucrose, 0.08 M KCl, 0.005 M $MgSO_4$, 0.035 M $KHCO_3$, 0.02 M nicotinamide and 0.02 M phosphate buffer, pH 7.5. Centrifugation was carried out at 15,000 × g in a Spinco Model L ultracentrifuge and the supernatant, containing microsomes and soluble fraction, used for incubation. Each flask contained 1 μmole/ml ATP, 10 μmoles/ml HDP, potassium salt, 220,000 counts/min of uniformly labeled L-[^{14}C]leucine* (approximately 0.06 μmole) and 2.2 ml of microsome suspension in a total volume of 2.5 ml. The flasks were incubated at 37° for 10 min, cooled to 0° and centrifuged at 105,000 × g for 1 h. The supernatant was then decanted off and the microsome pellet rinsed with 0.25 M sucrose, suspended in 0.25 M sucrose and the protein precipitated by the addition of cold 10 % TCA containing unlabeled leucine. The protein was washed by a standard procedure[5] and a weighed aliquot counted in a Nuclear micromil window counter. All counts were corrected to zero mass.

When RNA determinations were to be made, PCA was used as the precipitating agent and the precipitated protein was washed according to VOLKIN AND COHN[6]. The absorbency of the hot PCA extracts was determined in a Beckman spectrophotometer and compared with a standard RNA from yeast.

The protein content of the microsome pellet was determined gravimetrically.

The free leucine content of the liver was determined by the quantitative paper chromatographic method of KAY *et al.*[7], which actually measures leucine and isoleucine. The total free amino acid pool was determined on an aliquot of cell-free water extract of liver by the colorimetric method of ROSEN[8].

Insulin** added to the incubation flasks was present at a concentration of 0.25 unit/ml. It was added in 0.1 ml phosphate buffer at pH 8.5.

RESULTS

The addition of insulin to flasks containing 15,000 × g supernatant from normal rat liver produced no difference in the incorporation of leucine into microsomal protein when compared with control values. The average value for the specific activity of the microsomal protein was 550 counts/min/mg in these experiments. However, when insulin was injected into the animal 12 or 18 h prior to sacrifice, considerable increase in the specific activity of microsomal protein was attained, as compared with the control values. These specific activities showed some variability from animal to animal, more in the injected than in the control group (Table I). The average increase in specific activity in the injected group was 32 %.

Since a lowering of the free amino acid pool of the plasma following insulin injection has been demonstrated[9, 10], the possibility existed that the increase in amino acid incorporation observed following insulin injection might be due to a lesser dilution of the radioactive leucine by a smaller free leucine pool in the liver. However, since we found no consistent decrease in the free leucine pool of the liver following insulin injection, it is unlikely that the increased incorporation observed is due to such a factor. These values are recorded in Table II, and it can be seen from the standard

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* Obtained from Nuclear Chemical Corp., Chicago (U.S.A.).

** Glucagon-free crystalline zinc-insulin was kindly supplied by Dr. W. R. KIRTLEY of Lilly Research Laboratories.

TABLE I

INCORPORATION OF [^{14}C]LEUCINE INTO LIVER MICROSOMAL PROTEIN FROM CONTROL AND INSULIN INJECTED RATS

	Specific activity counts/min/mg	
Control	565	Average 550
Control	570	
Control	525	
Insulin injected	800	Average 752
Insulin injected	755	
Insulin injected	705	

Insulin injected rats received 40 units/kg body weight protamine-zinc insulin 18 h prior to sacrifice.

Each flask contained 10 µmoles/ml HDP, 1 µmole/ml ATP, 220,000 counts/min of [^{14}C]-leucine and 2.2 ml liver microsome suspension in a final volume of 2.5 ml.

TABLE II

FREE AMINO ACID POOL OF RAT LIVER (µmoles/g wet wt.)*

Total		*Leucine*	
Control	*Injected***	*Control*	*Injected***
61 ± 13	60 ± 16	0.87 ± 0.22	0.88 ± 0.29

* Values are the avarage of eight livers ± standard deviation.

** Injected animals received 40 units/kg body weight of protamine zinc insulin (Lilly) 12 h prior to sacrifice.

deviations that there is considerable variability in the total free amino acid pool and in the free leucine pool in both groups, possibly due to differences in food intake. It may be that the variation observed from animal to animal precluded demonstration of an effect of insulin on amino acid pool size in these experiments.

In order to demonstrate more conclusively that the stimulation of amino acid incorporation by prior insulin injection was inherent in the amino acid incorporating system, a series of experiments was carried out in which liver microsomes isolated from a normal animal were incubated in supernatant from an injected animal and *vice versa*. These results are shown in Table III, along with the leucine pool size when available, for comparison. It is apparent that the presence of both microsomes and supernatant from an injected animal is required for maximal incorporation. However, some stimulation is produced by incubating microsomes from a control with supernatant from an injected animal and *vice versa*. There are, therefore, at least two factors involved in this effect, one residing in the supernatant, the other in the microsomes themselves.

Since RNA (particularly that of the soluble fraction from liver) has been implicated in the process of protein synthesis[11], it was of interest to determine the RNA content of the 15,000 × *g* supernatant following insulin injection; however, insulin injection resulted in no change in RNA content.

Similarly, no difference was observed in the total protein content of the liver microsomes from control and insulin-injected rats.

TABLE III

INCORPORATION OF [^{14}C]LEUCINE INTO LIVER MICROSOMAL PROTEIN OF CONTROL AND INSULIN INJECTED RATS AFTER INCUBATION IN SUPERNATANT FROM EITHER LIVER

	Specific activity (counts/min/mg protein)				*Liver leucine pool µmoles/g wet weight*	
Microsomes	*"Normal"*		*"Injected"*		*Normal*	*Injected*
Supernatant	*"Normal"*	*"Injected"*	*"Normal"*	*"Injected"*		
	510	640	630	790		
	350	565	490	735		
	670	780	860	900	0.75	0.99
	475	475	575	645	0.80	0.70
	535	665	650	685	0.63	0.66
Average	508	625	647	751*		

Flasks contained 10 µmoles/ml. HDP, 1 µmole/ml. ATP, 220,000 counts/min [^{14}C]leucine and 2.2 ml of microsome suspension in a total volume of 2.5 ml.

Injected animals received 40 units/kg body weight protamine-zinc insulin 12 h prior to sacrifice.

* $P < 0.05$.

DISCUSSION

The lack of effect of insulin on amino acid incorporation, when the hormone was added to the isolated system, is in general agreement with observations of other workers (see the review by STADIE[12]) who have failed to observe an effect of insulin in a cell-free system.

Since the stimulation of amino acid incorporation into microsomal protein observed in livers from rats previously treated with insulin is of the order of 35–40 %, it is rather unlikely that small differences in leucine pool size, escaping detection in these experiments, are responsible for a significant part of this stimulation. On the other hand, there is a consistent increase in incorporation observed when the liver supernatant from insulin-injected rats is used in experiments with microsomes from normal rats. This suggests the possibility of some effect on the amino acid-activating system which is known to be present in the supernatant fraction of rat liver[13]. This effect is not mediated via an alteration in the RNA content, but a change in RNA structure is, of course, not ruled out. SACHS AND WAELSCH[14] have reported the presence in liver supernatant of a factor which is required for the stability of microsomes, and the possibility exists that this factor may be influenced by insulin.

In addition to its effect on the supernatant fraction, an important factor in the insulin-mediated stimulation of microsomal amino acid incorporation is the microsome itself, and it appears likely that this involves some alteration in the structure of the ribonucleoprotein molecules. It has been hypothesized that an alteration in microsomal structure involving, however, an increase in RNA content, is responsible for the increased amino acid incorporation observed in regenerating rat liver[15]. The fact that changes in microsomal RNA or protein content have not been detected in these experiments leads one to surmise that alteration in the structural relationships existing in the microsome may be a possible cause of the changes in incorporating ability found.

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NOUVELLES DONNÉES PHYSIQUES SUR LA GONADOTROPINE CHORIALE HUMAINE

RENÉ GOT ET ROLAND BOURRILLON

Laboratoire de Chimie Biologique, Faculté de Médecine, Paris (France)

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SUMMARY

Physical properties of human chorionic gonadotropin

The main physical properties of a pure human chorionic gonadotropin, with a titre of 12,000 I.U./mg have been established as follows: isoelectric pH: 2.95, sedimentation constant: 2.7 S, diffusion coefficient: $8.2 \cdot 10^{-7}$ cm^2/sec, partial specific volume: 0.727, extinction coefficient for a concentration of 1 mg/ml and a thickness of 1 cm: 0.425 ($\lambda = 278$ mμ), specific rotation: $-68°$. Applying Svedberg's formula, a molecular weight of 30,000 is found, a value confirmed by the diffusion of light; finally, the frictional ratio is 1.28. These new findings are discussed.

INTRODUCTION

Les propriétés physiques de la gonadotropine choriale humaine auxquelles on se rapporte actuellement, sont celles établies par GURIN *et al.*[1] et par LUNDGREN *et al.*[2] en 1939 et 1942. Aucune donnée nouvelle n'est venue confirmer ou compléter ces résultats.

Une méthode de préparation originale[3] nous a permis d'obtenir une hormone dont la pureté a été montrée par divers critères: électrophorèses libre et sur amidon, solubilité, sédimentation, diffusion, immunoélectrophorèse[4]. Les principales propriétés physiques de ce produit ont alors été déterminées: elles s'avèrent différentes de celles de la préparation de GURIN.

TECHNIQUE EXPÉRIMENTALE

Préparation hormonale

Toutes les déterminations ont été effectuées sur la préparation obtenue par une méthode de purification originale[3]. Son activité biologique est de 12,000 U.I./mg; sa teneur en azote est de 10.5 %.

Les concentrations des solutions utilisées dans les différentes mesures étaient obtenues par pesés du produit hormonal désséché. Toutes les solutions ainsi préparées étaient dialysées durant 18 h contre le solvant, avant l'expérience envisagée.

Détermination de la mobilité électrophorétique

Les électrophorèses en veine liquide ont été effectuées dans l'appareil de Tisélius (Perkin-Elmer, modèle 38), à 0°. Trois tampons ont été utilisées:

Véronal sodique–acétate de sodium–HCl	pH 8.6
Acétate de sodium–acide acétique	pH 4.5
Glycocolle–HCl	pH 1.5 à 3.5

La force ionique était toujours 0.1. Les concentrations en hormone variaient entre 0.5 et 1 %. Après la mesure de la conductivité de la solution protéique, le calcul des mobilités était effectué sur les clichés de la branche descendante.

Sédimentation

Les ultracentrifugations ont été effectuées dans une ultracentrifugeuse Spinco (modèle E), tournant à 59,780 rev./min (260,000 × *g*). La température était toujours de 20° C ± 0.5°.

Les essais étaient effectués par série de trois: le premier, sur une solution protéique de concentration déterminée, les deux suivants, sur des dilutions successives à partir de la solution mère.

Deux solvants ont été utilisés: un tampon phosphate $M/15$ de pH 7.3 et une solution 0.1 M de chlorure de sodium.

Diffusion

Les expériences de diffusion ont été réalisées dans la cellule de l'appareil à électrophorèse. La température était maintenue à 20°. Le déplacement de la frontière initiale était obtenu, à très faible vitesse, à l'aide d'un compensateur (2 cm de déplacement en 45 min). Le temps zéro était celui de la formation des frontières.

Deux solvants ont été utilisés: l'eau, avec une concentration protéique de 0.7 %, et une solution de chlorure de sodium 0.1 M, avec une concentration protéique de 0.5 %. La durée des essais était de 2 à 3 jours; ceux-ci étaient réalisés dans des conditions aussi stériles que possible, afin de préserver l'hormone de toute contamination. D'ailleurs, le maintien de l'activité biologique était une garantie de son intégrité.

Les clichés destinés aux mesures étaient agrandis. Trois méthodes de calcul ont été utilisées: la méthode des points d'inflexion, la méthode surface-ordonnée maximum, et la méthode des cordes[5]. Les mesures étaient effectuées sur des clichés correspondant à des durées suffisamment longues pour que le facteur de correction du temps zéro, défini par LONGWORTH[6], devienne négligeable.

Volume spécifique partiel

Les mesures du volume spécifique partiel étaient effectuées à 20° ± 0.05°, avec un pycnomètre de précision, d'un volume de 2 ml, dans lequel des tubes capillaires, divisés en 30 divisions identiques, permettaient de déterminer les variations de volume.

Le volume spécifique apparent (assimilé au volume spécifique partiel) était calculé par la formule suivante[7]:

$$\varphi = \frac{1{,}000}{C} \cdot \frac{d_1 - d}{d_1} + \frac{1}{d_1}$$

où C est la concentration en protéine (1 % environ), d_1 et d respectivement les densités du solvant et de la solution protéique. Le solvant utilisé était un tampon phosphate $M/15$ de pH 7.3. Une courbe d'étalonnage du pycnomètre avec le solvant était préalablement tracée.

Pouvoir rotatoire

Le pouvoir rotatoire était mesuré dans un polarimètre à lame demi-onde, dans un tube de 5 cm, la radiation étant la raie D du sodium.

L'hormone était dissoute à la température du laboratoire dans l'eau distillée, à une concentration de 44 mg par ml.

Absorption dans l'u.v.

Le spectre d'absorption dans l'u.v. était tracé de 5 en 5 mμ avec un spectrophotomètre Jobin et Yvon.

Trois solvants furent utilisés: HCl 0.1 N, H_2O et NaOH 0.1 N. Les concentrations étaient de l'ordre de 500 μg d'hormone/ml.

RÉSULTATS

Mobilité électrophorétique et point isoélectrique

Les électrophorèses effectuées à neuf pH différents ont permis de tracer la courbe de mobilité en fonction du pH (Fig. 1). Cette courbe coupe l'axe des pH au voisinage de pH 3, avec un $du/dpH_0 = 1.05 \cdot 10^{-5}$.

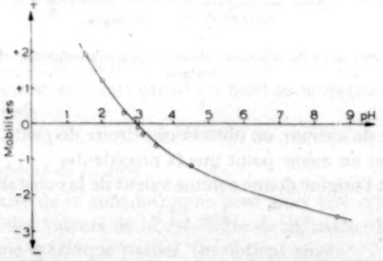

Fig. 1. Courbe de mobilité de la gonadotropine en fonction du pH (force ionique: 0.1).

Le point isoélectrique de la protéine est donc voisin de pH 3. Afin de préciser cette valeur, l'expérience suivante a été réalisée (Fig. 2): trois électrophorèses ont été effectuées avec une durée et une force du champ électrique identiques, dans le même tampon (glycocolle 0.1 *M*), mais, respectivement, à pH 2.9, 2.95 et 3. La polarité de l'appareil restant la même dans les trois cas, à pH 2.9 et à pH 3 la frontière hormonale migre de part et d'autre de la frontière de départ, alors qu'à pH 2.95 elle reste immobile.

On peut donc fixer le point isoélectrique de la gonadotropine à pH 2.95.

Fig. 2. Electrophorèses libres de la gonadotropine à proximité du pHi. Tampon glycocolle 0.1 *M*; 5.400 sec; E = 6.8 V/cm; pH 2.9; pH 2.95; pH 3.

Sédimentation

La variation de s_{20} en fonction de la concentration est représentée dans la Fig. 3. Avec le tampon phosphate pour solvant, on obtient une droite ayant pour équation:

$$s = 2.7\ \mathrm{S} - 0.07\ C$$

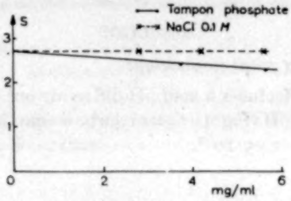

Fig. 3. Variation de la constante de sédimentation de la gonadotropine en fonction de la concentration.

Avec le chlorure de sodium, on obtient une droite de pente nulle qui coupe l'axe des ordonnées ($C = 0$) au même point que la précédente.

Cette ordonnée à l'origine donne comme valeur de la constante de sédimentation:

$$s = 2.7\ \mathrm{S}$$

Diffusion

Le Tableau I donne les moyennes de valeurs obtenues sur différents clichés, pris au bout de 30 à 55 h d'expérience, dans les deux solvants, par les trois méthodes de calcul citées. Remarquons la bonne concordance de ces valeurs dont la moyenne donne comme constante de diffusion:

$$D = 8.2 \cdot 10^{-7}\ \mathrm{cm^2/sec}.$$

TABLEAU I

CONSTANTES DE DIFFUSION DE LA GONADOTROPINE CHORIALE HUMAINE

Méthodes de calcul	*Solvant*	
	H_2O	*NaCl 0.1 M*
Points d'inflexion	8.19	8.35
Surface-ordonnée maximum	8.26	8.13
Cordes	8.12	8.05

Volume spécifique partiel

La Fig. 4 représente les courbes de poids de solvant et de solution en fonction du volume (ou du nombre de divisions du pycnomètre). La concentration en protéine était: $C = 9.91$ g/l. Les poids de solvant ont été mesurés pour des volumes correspondant aux divisions 15, 23, 34 et 49, les poids de solution, pour les divisions 15, 33.5 et 40.

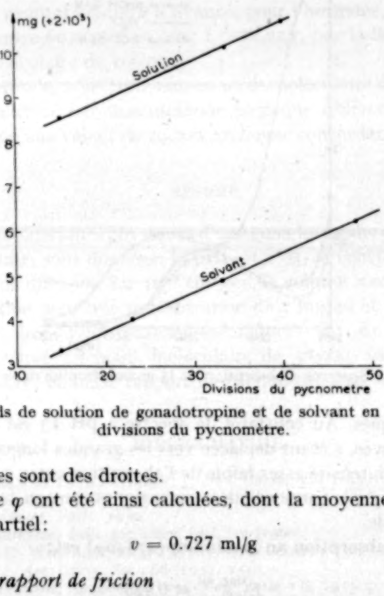

Fig. 4. Courbes de poids de solution de gonadotropine et de solvant en fonction du nombre de divisions du pycnomètre.

Les deux courbes sont des droites.

Trois valeurs de φ ont été ainsi calculées, dont la moyenne donne la valeur du volume spécifique partiel:

$$v = 0.727 \text{ ml/g}$$

Poids moléculaire et rapport de friction

Le poids moléculaire de la gonadotropine peut alors être calculé, par la formule de Svedberg, à partir des valeurs de la constante de diffusion, de la vitesse de sédimentation et du volume spécifique partiel. On obtient ainsi:

$$M = 30{,}000$$

Enfin, les déterminations précédentes permettent de calculer le rapport de friction:

$$f/f_0 = 1.28$$

valeur qui montre que la molécule n'est évidemment pas une sphère anhydre.

Pouvoir rotatoire

Dans les conditions expérimentales décrites, on observe une rotation de: — 1° 30′, soit un pouvoir rotatoire spécifique:

$$[\alpha]_{20}^{D} = -68°$$

Spectre d'absorption dans l'u.v.

La Fig. 5 montre les trois spectres obtenus. Les valeurs de l'absorption sont exprimées en coefficient d'extinction pour une épaisseur de 1 cm et une concentration de 1 mg/ml.

On remarquera que les spectres obtenus dans l'eau et dans HCl 0.1 *N* sont sensiblement identiques. Au contraire, le spectre à pH 13 est nettement différent, l'absorption, plus élevée, s'étant déplacée vers les grandes longueurs d'onde.

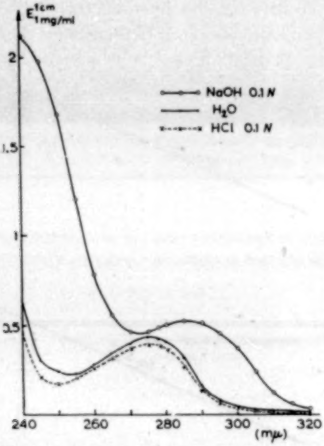

Fig. 5. Spectres d'absorption de la gonadotropine dans l'u.v.

Notons encore l'intensité assez faible de l'absorption entre 2,500 et 3,000 Å; il est donc vraisemblable que les aminoacides aromatiques sont en faible proportion dans la molécule hormonale.

Le coefficient d'absorption au maximum (278 mμ) est:

$$E_{1 cm}^{1 mg/ml} = 0.425$$

DISCUSSION

S'il existe plusieurs méthodes de préparation de la gonadotropine choriale, il n'existe qu'une étude physique de cette hormone: celle réalisée par GURIN ET LUNDGREN[1,2].

GURIN[1] a trouvé un point isoélectrique de 3.2–3.3, par une méthode de micro-électrophorèse. Il n'a affectué qu'une seule électrophorèse en veine liquide à pH 7, donnant une mobilité de — 4.8. Nous avons tracé une courbe de mobilité en fonction du pH, mettant en évidence une mobilité beaucoup plus faible (— 2.5 à pH 8.6) et permettant une détermination graphique du pHi: ce dernier est précisé par des électrophorèses effectuées à des pH l'encadrant de très près. Sa valeur est ainsi fixée à 2.95.

LUNDGREN[2] a donné pour la constante de sédimentation une valeur de 4.3 S. Cette valeur correspondait, non à l'extrapolation vers une dilution infinie, mais à une concentration de 1.8 % en hormone. Les ultracentrifugations effectuées dans deux solvants nous ont permis de tracer les courbes $s = f(C)$, qui sont deux droites dont l'ordonnée à l'origine ($C = 0$) est identique et égale à 2.7 S.

La constante de diffusion est également fort différente de celle qu'a donnée LUNDGREN: 8.2 au lieu de 4.4.

Quant au volume spécifique partiel, il est lui-aussi différent de celui de LUNDGREN (donné d'ailleurs comme imprécis, alors que la mesure de ce facteur est certainement plus précise que celle des autres constantes): 0.727 au lieu de 0.76, valeur qui semble trop élevée.

Partant de ces résultats, GURIN a avancé, pour l'hormone, un poids moléculaire minimum compris entre 60,000 et 80,000; LUNDGREN, par la formule de Svedberg, a calculé un poids moléculaire de 100,000.

Par la même formule, nous trouvons un poids moléculaire de 30,000. Cette valeur a d'ailleurs été confirmée par une méthode physique différente, la diffusion de la lumière*, qui a donné une valeur de 29,000, en bonne concordance avec la précédente.

RÉSUMÉ

Les principales caractéristiques physiques d'une gonadotropine choriale humaine pure, titrant 12,000 U.I./mg, sont données: le pHi est 2.95, la constante de sédimentation 2.7 S, la constante de diffusion $8.2 \cdot 10^{-7}$ cm^2/sec, le volume spécifique partiel 0.727, le coefficient d'extinction pour une concentration de 1 mg/ml et une épaisseur de 1 cm 0.425 ($\lambda = 278$ mμ), le pouvoir rotatoire spécifique —68°. En appliquant la formule de Svedberg, on trouve un poids moléculaire de 30,000, valeur confirmée par la diffusion de la lumière; enfin, le rapport de friction est 1.28. Ces résultats nouveaux sont discutés.

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THE SYNTHESIS OF METHIONINE BY ENZYMIC TRANSMETHYLATION*

IV. LIGHT SCATTERING STUDIES OF THE DEPOLYMERIZATION OF THETIN-HOMOCYSTEINE METHYLPHERASE

J. DURELL** AND R. F. STEINER***

Laboratory of Cellular Pharmacology, National Institute of Mental Health,
National Institutes of Health, Bethesda, Md.
Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md. (U.S.A.)

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SUMMARY

1. The parameters affecting the state of polymerization of horse liver thetin-homocysteine methylpherase were subject to quantitative study employing the measurement of light-scattering.

2. The pH and temperature, the concentration of the protein, and the presence of metal-chelating agents appeared to have virtually no effect on the extent of polymerization.

3. The rates of depolymerization produced by a number of disulfide cleaving agents were compared. The efficacy of various thiols roughly paralleled the acid strengths of their respective sulfhydryl groups, and for a given thiol the rate was increased by a rise in the temperature or pH.

4. Under conditions suitable for kinetic measurements, the depolymerization reaction slows progressively, and appears to cease before attaining completion.

5. The monomer mol wt. of the enzyme was approximately determined by sedimentation-diffusion and light scattering, and is estimated to be 180,000 ± 10%.

INTRODUCTION

Studies reported previously have shown that thetin-homocysteine methylpherase (THMenz) can be purified from horse liver, and that the purified protein preparation consists of a mixture of polymers of varying mol. wt. as evidenced by the ultracentrifugal sedimentation diagram[1]. When treated with any of a number of thiols, the protein dissociates into its constitutive monomeric units and then appears

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[*] Eighteenth paper in a series on enzymic mechanisms in transmethylation.

[**] Present address: Department of Psychiatry, Yale University School of Medicine, New Haven, Conn. (U.S.A.).

[***] The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

essentially homogeneous when subjected to electrophoresis or ultra-centrifugation. Various reagents were studied to determine, qualitatively, their effects on the state of polymerization of THMenz; the results suggested that the monomer units were bound together in the polymer by disulfide bonds[2]. It was the primary purpose of the experiments herein reported to confirm and extend these results by means of a quite different technique, the measurement of light scattering. Using this method, it has been possible to estimate quantitatively the relative efficacy of various reagents in effecting the depolymerization, as well as the dependence of the reaction on pH, temperature, and concentration. The results corroborate the suggestion that the polymer is bound together by disulfide bridges. In particular, no evidence was obtained for an alternative possibility, that the polymer units are held together through the binding of sulfhydryl groups to a bifunctional metallic cation, as occurs in the dimerization of mercaptalbumin in the presence of mercuric ion[3].

MATERIAL AND METHODS

Protein concentrations were determined spectrophotometrically at 280 mμ. The previously estimated[2] value of 1.21 for the extinction coefficient ($1\ g \cdot l^{-1}\ cm^{-1}$) of THMenz was used for the calculation.

Dimethylacetothetin (DMAT) and the optical isomers of homocysteine were prepared as previously described[1]. The other reagents were obtained commercially, and recrystallized when necessary.

The preparation of THMenz was carried through the electrophoretic purification[1]. Subsequent steps were at 3° unless otherwise stated. About 10 ml of a 1 % solution was then dialyzed for 72 h versus several changes of 0.1 M KCl, and subsequently saturated with K_2HPO_4 by addition of the solid salt. The insoluble protein was separated by centrifugal flotation and dissolved in sufficient 0.05 M KH_2PO_4 to provide a protein concentration of about 1 %. The final pH was adjusted to about 7. It was again dialyzed for 72 h against several changes of 0.2 M NaCl and subsequently frozen and stored at −20° until used. Ultracentrifugal examination at this stage revealed the protein to be very highly polymerized, less than 10 % appearing in the monomer peak.

In preparation for a series of experiments, the material was thawed, and about 10 ml of a 1 to 6 dilution in 0.2 M NaCl was prepared. This solution was repeatedly centrifuged in a Servall SS-1 at 14,000 rev./min until constant scattering properties (of a dilution) were attained. Appropriate dilutions with pre-centrifuged buffer were made for each experiment (0.20 M NaCl and 0.01 M potassium phosphate unless otherwise stated). The weight average mol. wt. of this material was about 2,000,000.

Light scattering was determined with a commercial light scattering photometer furnished by the Phoenix Precision Instrument Company. The method of absolute calibration and the details of measurement have been described elsewhere[4]. The light intensity at 45°, 90°, and 135° was determined with the use of a cylindrical cell with flattened entrance and exit windows. All measurements were made at either 26° or 6°, and the instrument was transferred as a unit into rooms thermostated at these temperatures. Usually a concentrated solution of the reagent to be studied was clarified by centrifugation, and at zero time in each experiment, 1 ml of the reagent was added to 30 ml of the diluted THMenz solution. Each set of runs included a control to which 1 ml of buffer was added.

The sedimentation coefficients of solutions of THMenz were determined with a Spinco Model E ultracentrifuge.

The diffusion coefficient of the depolymerized enzyme was determined in a Spinco electrophoresis-diffusion apparatus equipped with interference optics. A single experiment with a duration of 72 h at a protein concentration of 0.4 % was performed. The calculation followed closely the procedure recommended by LONGSWORTH[5].

RESULTS

A. *The mol. wt. of the monomer*

Light scattering and sedimentation-diffusion measurements were employed to determine the mol. wt. of THMenz monomer. The extrapolated sedimentation coefficient, S_{20}, in 0.05 M phosphate, pH 6.7, was $8.15 \cdot 10^{-13}$. When this was combined with the diffusion coefficient, D_{20}, of $4.3 \cdot 10^{-7}$, a mol. wt. of $1.84 \cdot 10^5 \pm 10\%$ was obtained, assuming a partial specific volume of 0.750.

Light scattering measurements at pH 6.7 upon a solution of THMenz in the presence of a large excess (0.74 g/l) of DL-homocysteine yielded, after repeated clarification to constant scattering properties, an apparent mol. wt. of $1.75 \cdot 10^5$ assuming a value of H, the Debye constant, equal to $1.0 \cdot 10^{-5}$. Further addition of homocysteine was without effect. Thus light scattering and sedimentation are in fair agreement as to the monomer mol. wt. However, it is not meant to claim the highest precision for the above figures, as they are based upon too few measurements.

B. *The effect of physical parameters on the degree of polymerization*

pH: The dilution of THMenz with buffers ranging in pH from 6.6 to 9.2 had no effect on the light scattering. It can be concluded, therefore, that in the range studied, pH, *per se*, had no significant effect on the degree of association of the protein, in the absence of added depolymerizing reagents.

Concentration: The light scattering as a function of the protein concentration was studied by making dilutions over the range of concentration from 0.04 to 0.16 g/l. No change in mol. wt. with dilution was noted in this concentration range.

Temperature: The light scattering of several polymerized THMenz solutions were compared at 6° and 26°. Only a marginal decrease in turbidity, which was never greater than 6 %, was observed at the higher temperature.

C. *The depolymerization reaction*

In all the experiments to be discussed subsequently, the controls were essentially negative; *i.e.*, the observed changes in mol. wt. in the absence of added reagent were negligibly small (< 5 % after 24 h).

Chelating agents: Table I shows the effect of large concentrations of the metallic binding agents Versene and 8-hydroxyquinoline. Only slight decreases in turbidity of THMenz solutions were noted, even after 24 h.

Disulfide cleaving agents: In contrast to the above, sodium sulfite and all the thiols tested produced significant depolymerization at much lower concentrations. Fig. 1 compares the relative efficacy of a number of reagents under similar conditions.

Marked differences in the rates of depolymerization were observed, the order of effectiveness being: β-mercaptoethylamine > cysteine > L-homocysteine > glutathione. Sodium sulfite at pH 6.6 compared in efficacy with glutathione, the least effective thiol. The rate of depolymerization with D-homocysteine was also studied

TABLE I

EFFECT OF CHELATING AGENTS ON THE POLYMERIZATION OF THETIN-HOMOCYSTEINE METHYLPHERASE

Solution of 0.078 g/l of THMenz was allowed to stand at 6° and pH 8.0 in the presence of the stated reagents for 24 h. The initial scattering was determined before the addition of the chelating reagent and was corrected for the subsequent dilution.

Reagent	*Concentration* $M \times 10^3$	τ^{0*} $(\times 10^3)$	τ^{*} $(\times 10^3)$	$\left(\frac{M_w}{M_w^0}\right)^*$
Versene	7.9	1.53	1.39	0.91
8-Hydroxyquinoline	1.2	1.52	1.37	0.91
None	—	1.52	1.46	0.96

* τ = Excess turbidity observed at 90°; M_w = calculated weight average mol. wt.; superscript "o" refers to measurements before the addition of the chelating agent.

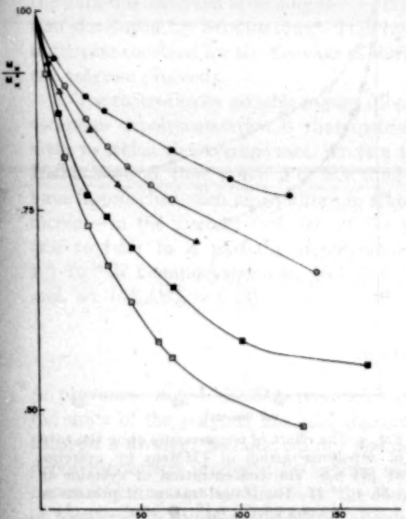

Fig. 1. The rates of depolymerization of thetin-homocysteine methylpherase when treated with various disulfide cleaving agents. All solutions contained 0.093 g/l of THMenz in 0.2 M NaCl, 0.01 M PO_4, pH 6.6. The temperature was 6.1°. The concentration of reagent was $2.86 \cdot 10^{-4}$ M except in the case of sodium sulfite, which was $5.72 \cdot 10^{-4}$. The relative weight average mol. wt. is plotted against time for each reagent. ● Glutathione; ⊙ L-homocysteine; ■ cysteine; ⊡ β-mercaptoethylamine; △ sodium sulfite.

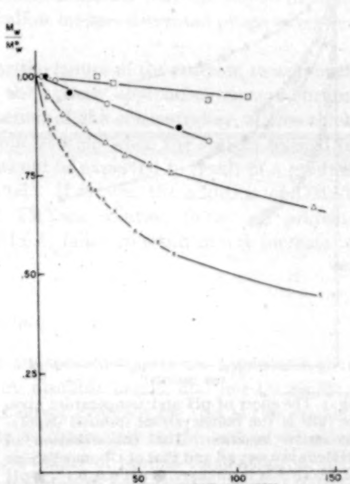

Fig. 2. The effect of varying L-homocysteine or protein concentration upon the rate of depolymerization. Conditions were otherwise identical with those of Fig. 1. ⊡ $0.057 \cdot 10^{-3}$ M L-homocysteine; 0.080 g/l THMenz; ⊙ $0.143 \cdot 10^{-3}$ M L-homocysteine; 0.045 g/l THMenz; ● $0.143 \cdot 10^{-3}$ M L-homocysteine; 0.161 g/l THMenz; △ $0.286 \cdot 10^{-3}$ M L-homocysteine; 0.093 g/l THMenz; × $2.71 \cdot 10^{-3}$ M L-homocysteine; 0.080 g/l THMenz.

and found to be slightly less than that observed with the L-isomer, but greater than that observed for glutathione; it has not yet been determined whether the difference is due to a difference in purity of the optical isomers employed.

Effect of concentration: Fig. 2 shows the rate of depolymerization of THMenz over a 50-fold range of L-homocysteine concentration; there is a marked increase in rate with increasing thiol concentration. On the other hand, in the two experiments in which the concentration of thiol did not vary, but the concentration of THMenz varied over a 4-fold range, the data are superimposable. Other disulfide cleaving agents behave similarly.

Effects of pH and temperature: Fig. 3 summarizes the rate of depolymerization obtained with a fixed concentration of L-homocysteine when the pH and temperature are varied. It can be seen that the rate of depolymerization increases markedly with increases of either of those parameters. Fig. 4 shows the effect of temperature on the cysteine induced depolymerization; the results resemble those obtained with homocysteine.

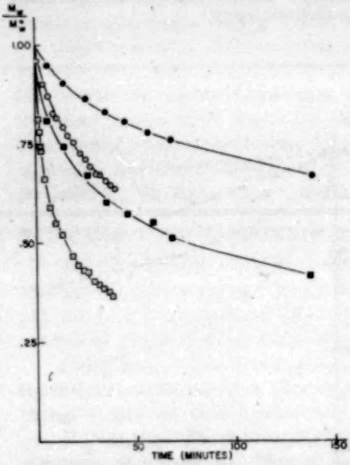

Fig. 3. The effect of pH and temperature upon the rate of the homocysteine induced depolymerization reaction. The concentration of THMenz is 0.093 g/l and that of L-homocysteine is $2.86 \cdot 10^{-4}$ *M* in all cases. ● pH 6.6, 6.1°; ■ pH 7.7, 6.1°; ⊙ pH 6.6, 26.1°; ☐ pH 7.7, 26.1°.

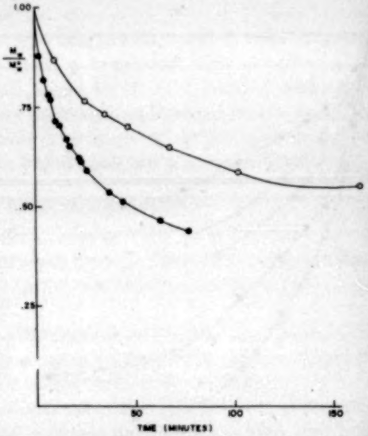

Fig. 4. The effect of temperature upon the rate of depolymerization of THMenz by cysteine at pH 6.6. The concentration of cysteine is $2.86 \cdot 10^{-4}$ *M*. The concentration of protein is 0.093 g/l. ⊙ 6.1°; ● 26.1°.

Extent of reaction: As shown in Section A, by choosing favorable conditions, it is possible to completely depolymerize THMenz to the monomer in the presence of a large excess of sulfhydryl reagent. Under the conditions employed for the kinetic experiments reported above, the reactions slow progressively and appear virtually to have stopped much before the monomer mol. wt. is approached. Table II shows the effect of prolonged standing at 6° with various thiols. The degree of depolymerization attained roughly parallels the initial rates of the reaction under the various conditions.

TABLE II

EXTENT OF DEPOLYMERIZATION ATTAINED AFTER PROLONGED STANDING

Reagent	*Concentration* $M \times 10^3$	*pH*	$\frac{M_w}{M_w^0}$	*Time (h)*
L-Homocysteine	0.14	6.7	0.44	20
L-Homocysteine	0.29	6.7	0.37	20
L-Homocysteine	0.29	7.7	0.23	20
β-Mercaptoethanolamine	0.29	6.7	0.21	20
Cysteine	0.29	6.7	0.35	23.5
Glutathione	0.29	6.7	0.47	23.5

Solutions used for the kinetic studies represented in Figs. 1, 2, and 3 were allowed to stand for the indicated time at 6.1° and the turbidity was measured. In general, the reactions had not quite ceased, but had become extremely slow.

It is to be noted that were depolymerization complete, M/M_0 would be expected to approach a value less than 0.1, which is considerably below any of the values actually observed. To confirm the apparent tendency of the reaction to slow before completion, the data was analyzed according to the statistical theory for condensation polymerization developed by STOCKMAYER[6]. This treatment indicated that the apparent first-order rate constant for the cleavage of the disulfide bridges decreased progressively as the reaction proceeds.

One theoretically possible explanation for the failure of the reaction to approach complete depolymerization is that instead, the system approaches an equilibrium state in which the average mol. wt. is a function of the concentration of free thiol. If this were so, then removal of free thiol from solution when the system seemed to have approached such an equilibrium state would be expected to result in a gradual increase in the average mol. wt. of the protein. However, the addition of DMAT ($1.0 \cdot 10^{-3}\,M$) to a partially depolymerized TMTenz solution (0.147 g/l protein; $1.3 \cdot 10^{-4}\,M$ L-homocysteine for 36 h at 6°, pH 6.8) failed to result in any increase in mol. wt. ($M_w/M_w^0 = 0.45$).

DISCUSSION

As previously noted, the data reported herein are consistent with the hypothesis that the units of the polymer are held together by disulfide bonds, and not by binding through a divalent metallic cation. The most direct evidence for this is the marked depolymerization produced by sodium sulfite as contrasted to the lack of effect of metal chelating agents. Moreover, the complete failure of protein concentration, pH, or temperature to affect the degree of polymerization to an important extent speaks in favor of disulfide bonds. The various depolymerizing agents would then owe their efficacy to the ability they all share, the cleavage of disulfide bonds. No data relevant to the mechanism of formation of these disulfide bonds is considered, and the question as to whether they are formed by interchange reactions or by direct oxidation of the sulfhydryl groups of two monomer units remains unanswered.

The most striking aspect of the kinetic results is that the order of effectiveness of various thiols in promoting depolymerization parallels the relative acid strengths of their sulfhydryl groups[7]. The sole exception is that an inversion occurs for the cases of glutathione and homocysteine. This is not surprising, however, because it has been observed that the reactivity of the sulfhydryl group of glutathione is generally low, and this has been alternatively attributed to the formation of a thiozoline ring[8] or to the formation of intramolecular hydrogen bonds[9]. In addition, it has been demonstrated that with a given thiol, the depolymerization is accelerated by raising the pH. These findings suggest that the effective agent in the depolymerization is the thiolate anion. Since we have not yet arrived at a satisfactory analysis of the kinetics, it is not possible to state whether the rate is actually proportional to the concentration of a given thiolate anion, and if so, whether the rate constant differs appreciably for different thiolate anions.

In the kinetic experiments described above, the molar concentration of free thiol varied from 10^2 to 10^3 times that of THMenz (calculated on the basis of the monomer mol. wt.). The depolymerization therefore, should occur without a significant change in concentration of the free thiol. If one assumes that all the disulfide cross linkages are equally reactive, then the reaction rate should be first-order with respect to the concentration of such cross-linkages. Since the statistical method of STOCKMAYER[6] allows the calculation of the concentration of cross-linkages from the weight average mol. wt. (for a given assumed functionality of the monomer unit), it is possible to determine whether such first-order kinetics actually apply. As stated above, when this is done, the apparent first-order rate constant is found to decrease progressively as the depolymerization proceeds.

Though it is possible that this kinetic result is simply an experimental artifact arising from the auto-oxidation of free thiol during the experiment, it may also indicate that the system approaches an equilibrium state or that its many cross-linkages are not all equally accessible to attack.

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ON THE BEHAVIOR OF THE *E. COLI* P_Z-"β-GALACTOSIDE SYSTEM" INTRODUCED INTO *SHIGELLA DYSENTERIAE*

MELVIN COHN*, E. LENNOX AND S. SPIEGELMAN

Department of Microbiology, Washington University School of Medicine, St. Louis, Mo. (U.S.A.)
Departments of Chemistry and Bacteriology, University of Illinois, Urbana, Ill. (U.S.A.)

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SUMMARY

The present experiments show that the ability to synthesize galactoside permease (Y) and β-galactosidase (Z) can be introduced into *Shigella dysenteriae* by transduction and sexual crosses from *Escherichia coli*. The resulting lac$^+$ *Shigella* do not have detectable amounts of P_Z, a protein immunologically related to Z and found in all *E. coli* strains tested. The data suggest that the P_Z locus cannot be closely linked to the β-galactosidase complex (y, z, i). The separation thus achieved demonstrates that synthesis of β-galactosidase is completely independent of the presence and formation of P_Z.

The β-galactosidase formed in *Shigella* is identical in all its tested immunological and enzymic properties to the enzyme found in *E. coli*. This is true whether the enzyme forming ability is transferred by either sexual or transductional recombination. Since the latter process introduces only a minute portion of the genome, the data support the contention that the specific properties of a protein molecule are determined by the relevant genetic information rather than the cytoplasm in which it happens to be operating.

The lac$^+$ Shigella clones isolated after transduction from constitutive *E. coli* donors are largely inducible and heterogenotes at the lac locus. To reconcile this finding with the known close linkage amongst the y, z and i loci, it is suggested the *Shigella dysenteriae* is genotypically i$^+$, the inducible and dominant allele of the i-locus.

INTRODUCTION

The ability of *E. coli* to utilize lactose for growth depends upon the presence of the "β-galactoside-system" which is composed of two distinct entities. One is a specific concentrating mechanism termed permease (Y), responsible for transporting the sugar into the cell. The other is the enzyme β-galactosidase (Z), which cleaves lactose to glucose and galactose[1-3]. The "β-galactoside-system" (Y and Z) in *E. coli* strains is present in two distinguishable states, inducible and constitutive. In inducible strains, the Y and Z activities are very low in the absence of external inducer, but high in its presence. With constitutive strains, external inducer is unnecessary for high levels of both Y and Z[4,5]. There are thus at least three phenotypic characters involved in the

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[*] Present address: Department of Biochemistry, Stanford University, Palo Alto, California.

"β-galactoside-system", Y, Z and inducibility. Of interest is that the corresponding genetic loci y, z, i are closely linked[6-8]. One other character appeared to be related. In all tested strains of *E. coli* and of *Sh. sonnei*, possessing or capable of acquiring β-galactosidase by mutation, there is a protein, P_Z, which cross-reacts with antibody to β-galactosidase[9,10]. The close structural similarity of P_Z to β-galactosidase implied by the immunological findings, suggested an ontogenetic relation between them. The obvious possibility that the constitutively synthesized P_Z is a precursor of the enzyme was eliminated by suitable labeling experiments[11,12]. It still, however, remained possible to entertain a number of other, less direct, types of relationships between these two proteins. It was clear that many of these could be put to the test by designing an experiment that would answer the following question: does the presence of a potential, or functioning, β-galactosidase-forming system insure the synthesis of the P_z protein? A negative answer would decisively eliminate many possible functional relations between P_Z and β-galactosidase formation. It is the primary purpose of the present paper to describe experiments pertinent to this issue. In the course of these studies data emerged relevant to an understanding of other features of the "β-galactosidase system" and these will be described and discussed.

The genetic characters controlling lactose utilization can be introduced *via* transduction with bacteriophage P1 and sexual crosses into strains of *Shigella dysenteriae* and *S. flexneri*[13,15] which are not known to acquire by mutation the ability to grow on lactose. Furthermore, since these strains do not possess the protein P_Z, they provide a system permitting a definitive resolution of the problem posed above. The data obtained in the course of the experiments to be described indicate that β-galactosidase can be synthesized in the absence of P_Z. They further suggest that *S. dysenteriae* possesses the inducible locus and is, therefore, $y^-z^-i^+$ in genetic constitution.

MATERIALS AND METHODS

Nomenclature

Following MONOD *et al.*[4,5], the galactoside-permease (Y) and the β-galactosidase (Z) will be symbolized with the indicated capital letters. These will be used only when permease or galactosidase are being measured directly either by kinetic or immunological methods. The genetic loci controlling the synthesis of the permease (y) and the β-galactosidase (z) will be denoted by the corresponding lower case letters. A third genetic locus, controlling inducibility (i), will also be discussed.

Bacterial strains

As lactose negative acceptors in both sexual crosses and transductions with bacteriophage P1, two strains of *Shigella dysenteriae* were used. Both were nonmotile and indole negative. One, (Sh) was wild type, and the other was a streptomycin resistant derivative (Sh/S). As lactose positive donors, three strains of *E. coli* were used. Wild type K-12 and K-15 (a K-12 derivative) were inducible ($y^+z^+i^+$) varieties. The third was a constitutive mutant, W-1317 ($y^+z^+i^-$). For certain experiments involving comparisons of levels of P_Z and Z, *E. coli* strain ML-30 was included.

The various mutant types may be classified as follows:

Previous designation[a]	*y*	*z*	*i*	*Growth on lactose*
Normal inducible	+	+	+	+
Normal constitutive	+	+	—	+
Cryptic inducible	—	+	+	—
Cryptic constitutive	—	+	—	—
Lac – absolute inducible	+	—	+	—
Lac – absolute constitutive	+	—	—	—

The cryptic inducible strains, while unable to utilize lactose, can be induced to form β-galactosidase by alkyl β-D-galactosides. These latter compounds can apparently enter the cell by non-permease routes unavailable to lactose. The designation, i^+, indicates inducible which is dominant over i^-, the constitutive allele[14]. The z^- strains do not show even the basal enzyme activities of non-induced inducible *E. coli*.

Virus strain

Bacteriophage P1kc[13] was used for all experiments and lysates were prepared on confluent lysis plates of the suitable host.

Media

All media, except where otherwise indicated, are as given in reference[13].

Procedure for transduction experiments[13]

Young broth cultures ($1 \cdot 10^8$/ml) of Sh or Sh/S were concentrated ten-fold by centrifugation, mixed with P1kc lysates (at a concentration of about $2 \cdot 10^{10}$ particles/ml and adsorption allowed to take place at 37°). The mixture was centrifuged, resuspended in about 0.1 volume of saline containing $2.5 \cdot 10^{-3}$ *M* Ca^{++} and aliquots spread on thick EMB agar plates containing 1 % lactose. Lactose positive papillae appeared in 2–3 days. Papillae were never seen on control plates of bacteria alone.

Procedure for crosses[15]

Young unaerated broth cultures of Sh/S and streptomycin sensitive substrains of *E. coli* K-12 were concentrated by centrifugation and resuspension to approximately 10^{10} cells/ml. Aliquots containing 10^9 cells of each type were spread together or alone on thick EMB agar plates containing 1 % lactose and 200 μg/ml dihydrostreptomycin. Sh/S alone does not yield lactose positive papillae. The *coli* strains alone usually yield no streptomycin resistant mutants at the level plated. On the mixed plates, however, approximately 50–150 lactose positive papillae appear after 3 days of incubation at 37°.

Procedure for testing isolates from crosses or transduction experiments

Papillae are picked from selection plates into saline, purified by several single colony isolations on EMB lactose plates, and analyzed for motility and indole production by standard methods. Scoring for ability to make β-galactosidase was done in the following manner: full grown, nutrient broth cultures of the isolates were each diluted 1:100 into a tube of nutrient broth and into a tube of nutrient broth containing the non-utilizable inducer methyl β-D-thiogalactoside (TMG) at a level of $2 \cdot 10^{-4}$ *M*.

They were then incubated at 37° for a period permitting a return to maximal turbidity. Comparison of enzyme level in the tubes with and without inducer was the basis for deciding whether the isolate is an inducible or constitutive enzyme former.

Procedure for enzyme assay

To assay a culture, it was first toluenized[16] and the suspension used as such. The assays for β-galactosidase were carried out with the chromogenic substrate *o*-nitrophenyl-β-D-galactoside under conditions previously described[3, 11]. The two procedures differ in the temperatures at which the reaction is carried out. The numbers given are all normalized to the lower temperature (28°). One unit of β-galactosidase, as defined here, hydrolyzes 1 mμmole of substrate/min at 28° pH 7.0 under saturating conditions of Na^+ and *o*-nitrophenyl β-D-galactoside[2].

Procedures for immunological assay

All cultures used for the immunological work were grown aerobically on inorganic salts medium with 0.1 % Difco yeast extract and 0.5 % succinate. The growth rate of a fresh isolate in this medium is about 0.2 divisions/h. However, after repeated passages and re-isolation, strains can be obtained with growth rates of 0.9–1.2 divisions/h. Strains which grow rapidly in the succinate medium are differentiated by adding succinate after their strain designation, (*e.g.*, *S. dysenteriae* Sh/S succ.). Succinate was used as a carbon source because it permits a high level of enzyme induction in *E. coli*[16].

TMG at a concentration of $2 \cdot 10^{-4}$ *M* was used as the inducer of β-galactosidase. A liter of an exponentially growing culture was centrifuged and washed with cold water, then extracted by grinding with an equal weight of alumina. The extract was centrifuged 1 h at 20,000 × *g* in the SS1 Servall centrifuge. Streptomycin was added to a concentration of 5 % to eliminate nucleic acids and some protein, which results in a more rapid formation of the specific precipitates upon addition of anti-β-galactosidase sera. Control recovery experiments show that streptomycin does not remove the P_Z or Z proteins. In some cases, in order to increase the sensitivity of the immunological assay, the P_Z and Z proteins were concentrated and purified three-fold by further fractionation between 0.25 and 0.40 saturated ammonium sulfate at pH 6.5.

The immunological methods used to assay for the P_Z and Z proteins have been described previously[9, 10]. The rabbit antiserum used to assay the P_Z was prepared by immunization with purified *E. coli* constitutive β-galactosidase. The resulting antiserum was fractionated to give a γ-globulin preparation.

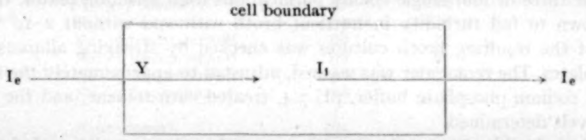
The immunological unit of Z is that amount of streptomycin treated crude extract expressed as mg N which will precipitate all of the anti β-galactosidase from 0.1 ml of the above γ-globulin preparation.

Similarly, the immunological unit of P_Z is that quantity which will precipitate the maximum amount of anti-β-galactosidase from 0.1 ml of the same preparation.

Procedure for analyzing for the ability to synthesize the β-galactosidase-Y system

The activity of the β-galactosidase synthesizing system is described in terms of the differential rate of synthesis, $P = \Delta Z/\Delta B$ where Z is β-galactosidase and B is bacterial protein nitrogen. Similarly, the ability of the culture to synthesize the Y system is measured as $\Delta Y/\Delta B$. The technique and interpretation of these measurements have been described previously[2,16].

The Y-system is the mechanism by which *E. coli* concentrates certain galactosides in the cell. The various properties of this system have been described by COHEN *et al.*[17], RICKENBERG *et al.*[18] and PARDEE[19]. These studies lead to the picture that a galactoside inducer, I_e, is concentrated *via* the Y system into the cell and then released by another mechanism. Thus, there is a steady state level of internal inducer I_1, established, which depends upon the ingoing and outgoing rates. The authors[17,18] just mentioned established an assay for the Y system based upon the ability of the cell to concentrate TMG labeled with ^{35}S *i.e.*, the steady state level of I_1. This assay has been modified as follows:



Instead of TMG, the noninducing analogue, β-β′-thio-digalactoside (TDG), was used as a substrate of the Y system. This analogue, as has been shown[1], has a much higher affinity than TMG for the Y system and, therefore, can be used even in the presence of TMG to assay for levels of Y. An aliquot of an exponentially growing culture was placed aerobically at 37° into a mixture consisting of $5 \cdot 10^{-4}$ *M* radioactive TDG (150,000 counts/min/ml) and 50 μg/ml of chloromycetin to stop growth. After 10 min the culture was passed through a HA millipore filter (diameter 0.94″) held in an iced stainless steel holder, and washed with 7 ml of ice water. The filter was air dried, glued to a copper planchet, and counted in a gas flow counter. In control experiments, the filters retained less than $1 \cdot 10^{-3}$ of the counts in the bacteria.

RESULTS

Repeated measurements of the transducing frequency for the lactose character into Sh gave a value of $1 \cdot 10^{-8}$ per adsorbed plaque-forming particle.

Stability of lactose positive Shigella *derivatives*[23,24]

The isolates derived from transductions and from crosses differ strikingly in the stability of the lactose positive character. From crosses, the isolates are stable after purification and seem to remain so indefinitely. On EMB lactose agar, they form very dark round colonies. Transduction experiments, on the other hand, yield two kinds of lactose positive colonies. The minority type resembles those derived from crosses. Some of these differ, however, in that although apparently stable through several single colony isolations, cultures kept on slants for about a month will contain lactose negative segregants. Re-isolations of a lactose positive colony from these slants yield cultures with an apparent stability similar to the original lac + type.

A second type of lactose positive isolate segregates on repeated single colony isolations into lactose negative colony formers and irregularly shaped, pale, sectored colonies. The ratio of sectored colonies to negative colonies from such an isolate is approximately 1.

The instability of the isolates in transduction experiments makes it essential to employ a nonutilizable inducer when measuring attainable levels of β-galactosidase activity. The use of lactose would complicate comparison of enzyme content since its presence gives a selective advantage to lactose positive cells, thereby altering their relative proportion in the population. Growth in the presence of TMG does not change the proportion of lac^+ to lac^-.

Constitutivity or inducibility in transductions and crosses

After three or four single colony purifications from selection plates, the isolates were grown to full turbidity in nutrient broth with and without $2 \cdot 10^{-4}$ *M* TMG. Purity of the resulting broth cultures was checked by streaking aliquots on EMB lactose plates. The remainder was washed, adjusted to approximately the same O.D. in 0.1 *M* sodium phosphate buffer, pH 7.4, treated with toluene, and the β-galactosidase levels determined.

With inducible donors the resulting recombinants are always inducible. The results of transduction experiments with P1kc, using a constitutive donor W-1317 (lac^+) are given in Table I. With this constitutive donor, 1 out of 61 isolates was constitutive, the balance being inducible. This single constitutive recombinant was a dark colony former and completely stable. Further, it was nonmotile, indole negative and lysogenic for P1kc.

TABLE I

THE β-GALACTOSIDASE LEVELS IN REPRESENTATIVE LAC$^+$ *Shigella* RECOMBINANTS MADE BY TRANSDUCTION FROM A CONSTITUTIVE DONOR

Isolates No. 1, No. 22, No. 221 are the dark colony type. The remainder of the recombinants are of the pale sectoring variety. Enzyme is expressed as mμmoles of substrate hydrolyzed/min/μg of bacterial protein nitrogen.

Isolate tested	*% lac$^+$ or lac$^+$ sectored segregants*	*Enzyme level*	
		With inducer	*Without inducer*
W-1317 (donor)	100	100	116
Sh (acceptor)	0	< 0.002	< 0.002
No. 1 (recombinant)	>99	33	0.04
No. 2 (recombinant)	30	1.6	< 0.002
No. 4 (recombinant)	50	2.7	0.02
No. 7 (recombinant)	60	3.7	0.02
No. 8 (recombinant)	40	4.2	< 0.002
No. 16 (recombinant)	40	3.1	< 0.002
No. 21 (recombinant)	80	17	0.02
No. 22 (recombinant)	100	70	0.01
No. 30 (recombinant)	90	0.7	< 0.002
No. 221 (recombinant)	100	54	86

Enzyme levels of representative recombinants derived from crosses are given in Table II. With a lac^+ constitutive donor, 12 out of 15 isolates were constitutive. This linkage is less than would be expected from the studies of JACOB AND MONOD[8, 14] on *coli* crosses and is partly accountable for if the inducible *Shigella* isolated in this cross were heterogenotes (see DISCUSSION). With an inducible parent, four isolates were tested and all were inducible. All recombinants were nonmotile and indole negative.

The enzyme levels in the lac^+ *Shigella* isolates are quite variable and in most cases

TABLE II

THE β-GALACTOSIDASE LEVELS IN REPRESENTATIVE LAC$^+$ *Shigella* RECOMBINANTS FROM SEXUAL CROSSES

In the upper part of the table, the recombinants listed are derived from a cross with a W-1317. In the lower part, the lac$^+$ parent is K-12 wild type. Enzyme is expressed as mμmoles of substrate hydrolyzed/min/μg of bacterial protein nitrogen.

Isolate tested	*Enzyme level*	
	With inducer	*Without inducer*
W-1317 (donor)	88	112
Sh/S (acceptor)	< 0.02	< 0.02
No. 6 (recombinant)	68	75
No. 11 (recombinant)	69	68
No. 19 (recombinant)	56	1.8
No. 30 (recombinant)	68	1.4
K-12 (donor)	162	1.2
No. 2 (recombinant)	42	< 0.02
No. 3 (recombinant)	36	< 0.02
No. 4 (recombinant)	40	< 0.02
No. 5 (recombinant)	43	< 0.02

low compared to in inducible *E. coli* grown in synthetic medium. This may in part be due to the inhibitory effect of nutrient broth on induction. Unfortunately, the growth requirements of these Shigella have not been worked out to the point where a usable defined synthetic medium is available.

The properties of the "β-galactosidase system" in Shigella dysenteriae

The introduction of Z without P_Z into *Shigella* raises the question whether any of the known properties of the β-galactosidase system would be affected. Since the ability to utilize lactose requires both the Y system and the β-galactosidase itself, it should be clear that the selection technique would pick up only those recombinants which had acquired both Y and Z. This, of course, was confirmed in the cases which were tested (Table III). Not only do the lactose positive *Shigella* possess both Y and Z, but the synthesis of these systems by *Shigella* is sensitive to the two inhibitors, glucose and phenyl-β-D-thio-galactoside[2, 5] in much the same way as the synthesis of Y and Z by *E. coli* (Table III).

The most sensitive tests employed to examine the identity of the galactosidases in *E. coli* and *S. dysenteriae* were immunological and a comparison of enzymic activities in the presence of various ions and inhibitors.

Immunological

The β-galactosidase introduced into *S. dysenteriae* is immunologically indistinguishable from that found in *E. coli*. The titration of both the constitutive and inducible β-galactosidases from *E. coli* and *Shigella dysenteriae* have been carried out using rabbit antisera prepared either against *E. coli* β-galactosidase (see Table III, last column) or against *S. dysenteriae* β-galactosidase (see Fig. 1). Using anti-*E. coli* β-galactosidase, the various enzyme preparations have the same enzymic activity/antigenic unit to within 10 % (Table IV). Fig. 1 shows in detail the reciprocal titration of various β-galactosidases with rabbit anti-*S. dysenteriae* Sh/S β-galactosidase.

TABLE III

COMPARISON OF THE INDUCTION PROPERTIES OF THE Y-SYSTEM AND β-GALACTOSIDASE IN *E. coli* AND *Shigella dysenteriae* STRAINS

The strains were induced in the succinate yeast extract medium containing $2 \cdot 10^{-4}$ *M* TMG. Glucose and phenyl-β-D-thio-galactoside (TPG) were added at 10^{-3} *M*.

Strain	*Additions*	$Z = \frac{\text{Enzymic activity}}{\mu g \text{ bacterial nitrogen}}$	$Y = \frac{m\mu \text{moles TDG accumulated}}{\mu g \text{ bacterial nitrogen}}$
E. coli K12	—	67	0.14
	Glucose	< 1	< 0.003
	TPG	< 1	< 0.003
E. coli ML 30	—	82	0.11
	Glucose	< 1	< 0.003
	TPG	< 1	< 0.003
Shigella dysenteriae	—	< 1	< 0.003
Sh/S	Glucose	< 1	< 0.003
Lac$^-$	TPG	< 1	< 0.003
Shigella dysenteriae	—	48	0.18
Sh/S Succ.	Glucose	< 1	0.021
Lac$^+$ by transduction	TPG	< 1	< 0.003
Shigella dysenteriae	—	< 1	< 0.003
Sh/S Succ.	Glucose	< 1	< 0.003
Lac$^-$ segregant	TPG	< 1	< 0.003
Shigella dysenteriae	—	53	0.31
Sh/S Succ.	Glucose	< 1	0.017
Lac$^+$ by cross with K12	TPG	< 1	< 0.003
Shigella dysenteriae	—	< 1	< 0.003
Lac$^-$ crossed with	Glucose	< 1	< 0.003
K12	TPG	< 1	< 0.003
Shigella dysenteriae	—	104	0.45
Lac$^+$ constitutive	Glucose	79	0.38
	TPG	94	< 0.003
Salmonella typhimurium	—	< 1	< 0.003
	Glucose	< 1	< 0.003
	TPG	< 1	< 0.003
Aerobacter aerogenes	—	57	0.22
	Glucose	2.3	< 0.003
	TPG	< 1	< 0.003

Enzymic

The inducible *S. dysenteriae* and *E. coli* enzymes are indistinguishable by a sensitive test of their enzymic properties[21] (see Table V). Furthermore, both enzymes are equally well inactivated by heat, Versene, and *p*-chloro-mercuribenzoate.

These data support the conclusion that the introduction of an *E. coli* enzyme forming system into *Shigella* yields the same protein.

Assays for material cross reacting serologically with β-galactosidase

The surprising observation is that introduction of the *E. coli* β-galactosidase into *Shigella* is not detectably accompanied by the presence of P_Z. At first this failure to

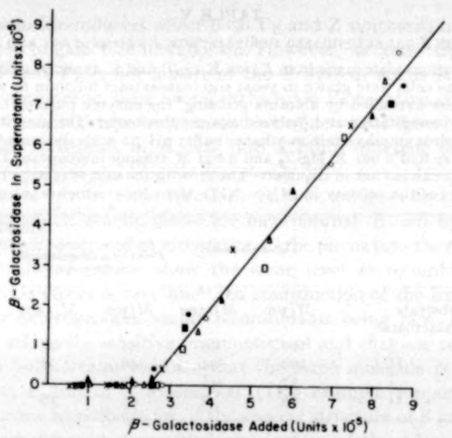


Fig. 1. The Titration of various β-galactosidase preparations with a Rabbit antiserum to the *Shigella dysenteriae* Sh/S succ. inducible β-galactosidase. β-galactosidase from: Sh/S constitutive ×—×; Sh/S lac$^+$ inducible by transduction ○—○; Sh/S lac$^+$ inducible by cross △—△; *E. coli* K15 (lac$^+$) ■—■; *E. coli* K12 (lac$^+$) ▲—▲.

TABLE IV

IMMUNOLOGICAL TITRATIONS FOR P_Z AND β-GALACTOSIDASE IN EXTRACTS OF VARIOUS *Escherichia coli* AND *Shigella dysenteriae* STRAINS

Note that Enzyme units are here expressed/mg of bacterial nitrogen.

Strain	*Treatment*	*Activity units (Z)/mg N*	*P_Z/mg N*	*Immunological units (E)/mg N*	*Activity/immunological unit of E*
E. coli ML 30	Noninduced	47	23	< 0.05	—
	Induced	$7.3 \cdot 10^4$	11	19	3800
E. coli K12	Noninduced	22	18	< 0.05	—
	Induced	$8.8 \cdot 10^4$	28	25	3500
E. coli K15	Noninduced	27	16	< 0.05	—
	Induced	$9.6 \cdot 10^4$	15	26	3700
Shigella dysenteriae					
Sh/S	Noninduced	< 5	< 1	< 0.05	—
Lac$^-$	Induced	< 5	< 1	< 0.05	—
Shigella dysenteriae					
Sh/S	Noninduced	< 5	< 1	< 0.05	—
Lac$^+$ by transduction	Induced	$1.2 \cdot 10^4$	< 5	4	3000
Shigella dysenteriae					
Sh/S succ.	Noninduced	26	< 1	< 0.05	—
Lac$^+$ by transduction	Induced	$12 \cdot 10^4$	< 5	31	3900
Shigella dysenteriae	Noninduced	< 5	< 1	< 0.05	—
Lac$^+$ by cross with K12	Induced	$2.4 \cdot 10^4$	< 5	7	3500
Shigella dysenteriae	Noninduced	< 5	< 1	< 0.05	—
Lac$^-$ crossed with K12	Induced	< 5	< 1	< 0.05	—
Shigella dysenteriae					
Sh/S succ.	Noninduced	< 5	< 1	< 0.05	—
Lac$^-$ segregant	Induced	< 5	< 1	< 0.05	—
Shigella dysenteriae					
Lac$^+$ constitutive	—	$8.9 \cdot 10^4$	< 1	25	3600

TABLE V

COMPARISON OF THE β-GALACTOSIDASES DERIVED FROM *Escherichia coli* AND *Shigella dysenteriae*

The enzyme preparations were made from *E. coli* K 12 (I) and *S. dysenteriae* Sh/S Succ. Lac + (II) by transduction. The cells were grown in yeast succinateextract medium to which 10^{-3} TMG was added. The cells were extracted by alumina grinding, the enzyme purified by streptomycin and ammonium sulfate precipitation and dialyzed against this buffer. The activities were carried out in *M*/50 Tris (trishydroxymethylaminomethane) buffer pH 7.2 with the ion added as the chloride. The reaction mixture had 0.001 *M* $MgCl_2$ and 0.002 *M* ethanol mercaptan. The temperature was 31°. Each assay was carried out in triplicate. The error in the assays is 8%. The values above are percentages of the activity in *M*/300 NPG *M*/10 Na+ which was assigned 100.

		Without inhibitor		*With inhibitor*			
				Phenyl-β-D-thiogalactoside M/500		*Methyl-β-D-thiogalactoside M/10*	
Concentration of substrate o-nitrophenyl β-D-galactoside		*M*/300	*M*/1500	*M*/300	*M*/1500	*M*/300	*M*/1500
M/10 Na^{+}	I	100	95	55	25	65	28
	II	100	94	49	32	64	23
M/10 K^{+}	I	57	41	43	31	31	23
	II	45	50	45	38	36	15

detect P_Z was thought to be related to the fact that in nutrient broth induction of β-galactosidase was poor. Therefore, lac^{+} *Shigella* strains were isolated which grew well in succinate—0.1% yeast extract—inorganic salt medium. However, even those isolates of *Shigella* which can make high levels of β-galactosidase yielded no detectable amounts of P_Z (see Table III). Thus, it would appear that a separation of P_Z and β-galactosidase has been accomplished. It should be noted that the assay for P_Z is not very sensitive and the lowest detectable level is about 5–10% of the maximum levels found in *E. coli*. Allowing for the insensitivity of the assay for P_Z, the experiment described in Table IV shows that P_Z is not part of the Y-system.

DISCUSSION

The experiments described in the present paper are relevant to a discussion of the following three problems; (a) the P_Z-β-galactosidase relationship; (b) the control of enzyme structure by a gene in a foreign cytoplasm; and (c) the nature of constitutivity.

The original hypothesis that P_Z and Z were related in their synthesis was based on a number of observations[9,10]. These included their immunological relatedness, the fact that induction of Z was correlated with a change in the P_Z level, and finally that, until that time, P_Z was always found in organisms which could synthesize Z following either mutation or induction or a combination of these two events. When it became clear that the P_Z was not a precursor of Z, the alternate hypothesis was proposed[12] that P_Z and E were made at a common site to account for the above findings. Our present results show that P_Z synthesis is not mandatorily coupled to the synthesis of Z. Furthermore, although only five *Shigella* lac^{+} isolates have been examined for P_Z, it is clear that the P_Z locus is *not* closely linked to the y – z – i complex.

It is not known whether P_Z synthesis, which is constitutive in *E. coli*, might not be inducible in *Shigella* in which case one can only conclude that we do not know the correct inducer. Within this limitation, the most likely hypothesis concerning the P_Z–Z relationship is that there are two distinct systems which make similar proteins.

The fact that galactoside inducers affect both P_Z and Z synthesis, implies that P_Z is in some way related to galactose metabolism. However, we know from the results of the present study that P_Z is not a part of the Y system nor is it involved in the utilization of galactose *per se*, since *Shigella* which are P_Z negative grow on galactose.

The situation described here involves the introduction of the z-cistron concerned with β-galactosidase synthesis in *E. coli* into a quite different organism *Shigella dysenteriae*. *Escherichia coli* and *Shigella dysenteriae* differ, not only by a whole host of growth requirements, fermentation reactions, surface antigens and by motility, but also by the way in which genetic pieces are incorporated. *E. coli* by *E. coli* crosses, or transductions into *E. coli*, lead to integration of the pieces into the recipient genome. *E. coli* by *S. dysenteriae* crosses show the same level of recombination, but the integration of genetic pieces is very low[15]. In transduction of the lac locus, one finds almost exclusively heterogenotes, stable recombinants being rare. In spite of these many differences, relatively sensitive immunological and enzymic comparison of the β-galactosidase in both strains indicate that the same molecule is formed whether synthesis occurs in *E. coli* or *S. dysenteriae*. (This example supports the one gene (cistron)—one enzyme hypothesis for, if the specific structure of β-galactosidase were controlled by many different genes, the introduction of the z-locus of *E. coli* into *S. dysenteriae* would not be expected to yield the same enzyme.)

The question of constitutivity raises some interesting relationships. Lactose utilization by *E. coli* is controlled by two closely linked loci, y and z. Whether or not these two loci are phenotypically expressed in the absence of an inducer, depends upon a third locus, i. In the i^+ state, the strains are inducible, whereas in the i^- state, they are constitutive for both the permease and the β-galactosidase.

LEDERBERG[6] and MONOD[5] have shown that the lac_1 locus corresponds to the y cistron and is closely linked to the lac_4 locus (lac^- absolute) which controls Z synthesis. All mutations shown to lack Z by direct measurement are in the same cistron (z)[8]. *S. dysenteriae* lacks both Y and Z and is assumed to be y^-z^-. Since lac^+ *Shigella* clones are picked from lactose plates selection is for the doubly transduced, y^+z^+. The i locus is an unselected marker. Thus, theoretically a test for the linkage of i to y-z is possible.

The original test of the question using sexual crosses in the K12 system indicated that the y-z and i loci were closely linked[7]. These findings have been confirmed here, by crosses into the *Shigella* system (Table II). Theoretically transduction provides a more sensitive test of linkage since the frequency of joint transduction of two markers falls off rapidly with the distance between them[13, 23]. Taken at their face value, the present transduction experiments (Table I) with constitutive donors and *Shigella* recipients would seem to suggest that the i locus is not tightly linked to the y-z complex.

This apparent contradiction between the results of recombination and those of transduction might have been simply ascribed to the greater sensitivity of the transductional test for linkage. However, similar transduction experiments with *E. coli* recipients show y - z - i to be closely linked[8].

What then is peculiar about the *Shigella* system? While it seems clear that *Shigella* recipients are y^-z^-, it is not known whether they are i^- or i^+. PARDEE, JACOB AND MONOD[14] have demonstrated that i^+ is dominant over i^- by showing that the zygote between Hfr ($y^+z^+i^+$) and F^- ($y^+z^-i^-$) makes enzyme in the absence of external inducer whereas the reciprocal zygote does not. This, and other evidence[22], leads to the conclusion that an inducible enzyme forming system is in a repressed state. With this result in mind, the sexual crossing and transductional linkage data can be reconciled with the assumption that *Shigella* is i^+.

It was pointed out here that most recombinants of *Shigella* from sexual crosses are stably lactose positive whereas those resulting from transduction are unstable, *i.e.*, constantly segregating lactose negative. The stable crosses of *E. coli* ($y^+z^+i^-S^s$) with *S. dysenteriae* $y^-z^-i^+S^r$ yield 80 % constitutives by crossing over between i and S. The unstable lac$^+$ transduced *Shigella* isolates can be considered as heterogenotes of the kind $\left(\frac{y^+z^+i^-}{-\ -\ +}\right)$ where a recessive i^- gene is in the presence of a dominant i^+ one. Thus, these isolates are phenotypically inducible. With a frequency of 1–2 % a stable constitutive recombinant is obtained by the same crossover which yields them in sexual recombination. The constitutive *Shigella* strain described in this paper is completely stable as would be expected.

It should be noted that the order, ($y^+z^+i^-$) has not been unambiguously established by the data presented here. It is, however, suggested by the fact that approximately 25 % of the lactose positive recombinants obtained from the sexual crosses between *E. coli* constitutives and *Shigella* were of the inducible type. This class emerges readily via a single x-over from the order noted above and would require a double x-over in a very small region if the i-locus were placed between y and z.

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THE NATURE AND MECHANISM OF ACTION OF UNCOUPLING AGENTS PRESENT IN MITOCHROME PREPARATIONS

W. C. HÜLSMANN, W. B. ELLIOTT* AND E. C. SLATER

Laboratory of Physiological Chemistry, University of Amsterdam (The Netherlands)

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SUMMARY

1. The uncoupling activity of mitochrome preparations was not affected by addition of $Na_2S_2O_4$, $K_3Fe(CN)_6$, CO or 10^{-4} *M* KCN, all of which bring about changes in the absorption spectrum of mitochrome.

2. The uncoupling activity was also not affected by precipitation of the protein by heating the solution or adding trichloroacetic acid.

3. The substance(s) responsible for the uncoupling activity could be completely extracted by organic solvents; the mitochrome remained in the water layer.

4. Purified preparations of cytochrome ($a + a_3$) or cytochrome *b* changed into mitochrome on standing at 4° for a few days. Simultaneously, there was an increase in the amount of uncoupling material which could be extracted from these preparations by organic solvents.

5. The uncoupling activity of an iso-octane extract of mitochrome resides in the unsaturated fatty acids, chiefly oleic and linoleic acids, which it contains.

INTRODUCTION

In 1952 Lardy[1] suggested that one of the mechanisms of the control of cell metabolism might be the presence in the cell of substances which uncouple oxidative phosphorylation. Lardy's idea could be extended by postulating that the uncoupling substance is not always present in an active form, but is liberated from a precursor under certain conditions. Indeed, freshly prepared liver mitochondria are not appreciably uncoupled, but on standing at room temperature a suspension develops many of the characteristics of freshly prepared mitochondria to which a typical uncoupling agent such as dinitrophenol is added, namely a decrease of the P : O ratio and of the P_i-ATP exchange reaction, and an increased ATPase activity. At the same time, structural damage is observed, particularly to the mitochondrial membranes. This suggests that during the "ageing" of the mitochondria, an uncoupling agent is produced, possibly from the mitochondrial membranes.

An uncoupling agent was first isolated from disintegrated, aged mitochondria

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Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

[*] On leave from the Department of Biochemistry, University of Buffalo, Buffalo, New York (U.S.A.)

by POLIS AND SHMUKLER[2] and PULLMAN AND RACKER[3]. The purified compound[2] was called mitochrome because it had the properties of a haemoprotein. Experiments will be reported in this paper confirming the uncoupling action of the mitochrome preparation. However, we have found that this cannot be ascribed to the haemoprotein itself but to components, extractable by organic solvents, present in the mitochrome preparations. Preparations of the cytochromes ($a + a_3$) and b, which yielded mitochrome on ageing, were also found to contain the uncoupling agents. A preliminary report of some of these findings has appeared[4].

METHODS

Reagents

The ATP and Tris were obtained from Sigma Chemical Co. $H_3{}^{32}PO_4$ was purchased from Philips Roxane (Holland), bovine serum albumin from Armour, and isooctane from Brocades-Stheeman & Pharmacia. EDTA, ethanolamine, sucrose, KCl, 2,4-dinitrophenol and most of the other chemicals used were Analar Reagent supplied by British Drug Houses, Ltd. Hexokinase was prepared from yeast as described by BERGER *et al.*[5] up to step 5. The water used to make up the solutions was double (glass) distilled.

Preparations

Rat-liver mitochondria were isolated in 0.25 M sucrose at 0–2° by the method of HOGEBOOM[6], as described by MYERS AND SLATER[7].

Mitochrome was prepared from whole beef liver according to the method of POLIS AND SHMUKLER[2].

Cytochrome (a + a_3) *and cytochrome* b preparations were made as described by ELLIOTT, HÜLSMANN AND SLATER[8].

Extracts in organic solvents of mitochrome or of cytochrome preparations were prepared by shaking 1 volume of the preparation with 3 volumes of the organic solvent at 20 ± 2° for 2 min (or for 1 min when the extraction was carried out twice). After centrifugation the organic layer was removed (or when an organic solvent was used which mixed with the water layer, the entire clear supernatant was removed), and the latter evaporated to dryness on a water bath, under a stream of nitrogen. The residue thus obtained was dissolved in 96 % ethanol.

Methods

ATPase activities were measured as described by MYERS AND SLATER[7]. 0.05 ml of a freshly prepared liver mitochondria preparation (0.5–1.0 mg protein) was added to an incubation medium of 1.45 ml containing, in addition to the reagents to be tested, 0.002 M ATP, 0.075 M KCl, 0.0015 M $MgCl_2$, 0.0005 M EDTA, 0.05 M sucrose and 0.05 M Tris-acetate buffer[7] or of Tris–ammonium-acetate buffer[7] of the desired pH. The reaction was carried out at room temperature (20 ± 2°). After 15 min incubation the reaction was stopped by the addition of 1.5 ml 10 % trichloroacetic acid. The tubes were centrifuged at 3000 rev./min for 5 min and phosphorus determined in the clear supernatant fluid by the method of FISKE AND SUBBAROW as modified by SUMNER[9].

Oxidative phosphorylation: P:O ratios were determined by the procedure of

SLATER AND HOLTON[10]. The reaction mixture contained 0.015 M KCl, 0.002 M EDTA, 0.02 M glucose, 0.03 M K phosphate, pH 7.0 or 7.5, 0.025 M Tris–acetate buffer[7], pH 7.0 or 7.5, 0.0001 M ATP, 0.005 M $MgCl_2$, 0.05 M sucrose, hexokinase (100–150 units[5]), 0.01 M glutamate or 0.02 M succinate as indicated, and liver mitochondria (3–4 mg protein). The final volume was 1 ml, the reaction temperature 25° and the reaction time 25 min.

P_i-ATP exchange reaction: This reaction[11, 12] was carried out in centrifuge tubes at room temperature (20 ± 2°). The tubes contained (in a volume of 1.45 ml) 0.065 M KCl, 0.003–0.004 M $MgCl_2$, 0.001 M EDTA, 0.006 M ATP, 0.067 M Tris–acetate buffer[7] of the desired pH, and 0.007 M phosphate buffer of the same pH containing ^{32}P. The reaction was started by the addition of 0.05 ml liver mitochondria (in 0.25 M sucrose) and after 15 min stopped by the addition of 1.5 ml cold 10 % trichloroacetic acid. After centrifugation at 3000 rev./min, the amount of radioactivity in the ATP was determined in a sample of the clear supernatant fluid after separation of the inorganic phosphate from the organic phosphate (ATP) by the method described by NIELSEN AND LEHNINGER[13]. The counting apparatus consisted of a Geiger-Müller tube + automatic scaler (Philips). The samples (0.5 ml) were counted wet in aluminium dishes. After calculation of the total number of counts/min in the ATP, this value was divided by counts/min/µmole inorganic phosphate, yielding the number of µmoles of inorganic phosphate exchanged with the phosphate of ATP. After correction for the breakdown of ATP due to ATPase (amounting to not more than 2 %) the exchange was calculated as µmoles/mg protein/h. The amount of mitochondrial protein was generally about 1.0 mg, which gave, under the conditions described, an almost linear exchange with time.

Protein was determined by the biuret method[14] standardized with serum albumin.

RESULTS

Uncoupling activity of mitochrome

Mitochrome, prepared from whole beef liver by the procedure of POLIS AND SHMUKLER[2], was found to have the same spectral properties as described by these authors (see ELLIOTT *et al.*[8]). Table I shows that we were also able to confirm the uncoupling effect of this preparation on oxidative phosphorylation and the stimulatory effect on the latent ATPase of freshly prepared mitochondria. It also strongly inhibited the P_i-ATP exchange reaction of mitochondria (Table II).

Table II shows that substances which alter the absorption spectrum of mitochrome, namely $Na_2S_2O_4$, $K_3Fe(CN)_6$, CO and KCN*, had no effect on the uncoupling activity of mitochrome, which strongly indicated that the haem was not responsible for the uncoupling activity. Moreover, precipitation by heat or by trichloroacetic acid did not destroy the uncoupling activity (Table III). The supernatants obtained by either treatment were inactive. Alcohol, acetone, butanol or isooctane extracts were active. Isooctane was found particularly suitable for extracting the active principle from mitochrome preparations, since a single extraction caused no precipitation of protein. Fig. 1 shows that after extraction of the uncoupling activity into the

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* In agreement with other workers, a higher concentration of cyanide (10^{-3} M) caused 48 % inhibition of the exchange reaction.

TABLE I

EFFECT OF THE MITOCHROME PREPARATION ON OXIDATIVE PHOSPHORYLATION AND LATENT ATPASE OF LIVER MITOCHONDRIA

Oxidative phosphorylation was carried out at pH 7.0 with succinate as substrate. The ATPase reaction was carried out at pH 6.5.

Additions	*P:O*	*ATPase µatoms P/mg protein/h*
None	1.52	0.3
Mitochrome (0.66 mg protein)	0.01	
Mitochrome (0.33 mg protein)		4.0

TABLE II

EFFECT OF A MITOCHROME PREPARATION AND OF SUBSTANCES REACTING WITH HAEM COMPOUNDS ON THE P_i-ATP EXCHANGE REACTION OF LIVER MITOCHONDRIA

The pH of the incubation medium was 7.0.

Additions	*µatoms P exchanged/mg protein/h*		*Inhibition %*
	No mitochrome	*Mitochrome (0.16 mg protein)*	
None	4.1	0.4	90
$Na_2S_2O_4$ (10^{-3} *M*)	3.5	0.2	94
$K_3Fe(CN)_6$ (10^{-2} *M*)	4.3	0.2	95
KCN (10^{-4} *M*)	3.7	0.4	89
CO (100% in gas phase) + $Na_2S_2O_4$ (10^{-3} *M*)	3.5	0.2	94

TABLE III

EFFECT OF VARIOUS FRACTIONS OF A MITOCHROME PREPARATION ON THE P_i-ATP EXCHANGE REACTION

The pH of the incubation medium was 7.0.

Additions	*µatoms P exchanged/mg protein/h*
None	4.1
Mitochrome (0.33 mg protein)	0.2
Mitochrome, first precipitated with trichloroacetic acid, then neutralized with KOH and rehomogenized	0.4
Mitochrome, heated at 100° for 10 min (homogenized to make a fine suspension)	0.3
Clear supernatant fluid (neutralized with KOH) of mitochrome precipitated with trichloroacetic acid	3.4
Clear supernatant fluid of heat-denatured mitochrome	3.5
Ethanol (2.4% final concentration)	3.9
Ethanol extract of mitochrome	0.3
Acetone extract of mitochrome	0.2
Isobutanol extract of mitochrome	0.1
Isooctane extract of mitochrome	0.2

isooctane layer, the spectrum of mitochrome remained unchanged in the water layer. Table IV illustrates that one extraction with isooctane is sufficient nearly completely to remove the uncoupling activity, while the activity of the isooctane extract, like that of the mitochrome preparation itself[2], is counteracted by the addition of serum albumin.

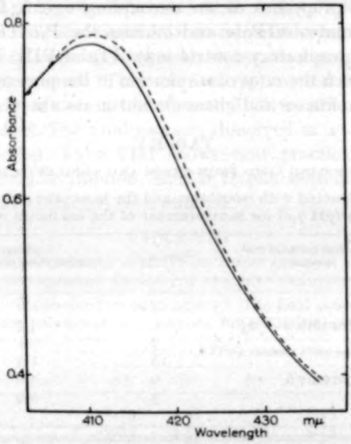

Fig. 1. Effect of extraction with isooctane on the absorption spectrum of mitochrome. ----, unextracted; —— extracted. Equal amounts of unextracted and extracted mitochrome inhibited the P_i-ATP exchange reaction by 94 % and 34 %, respectively.

TABLE IV

EFFECT OF ISOOCTANE EXTRACTION ON THE UNCOUPLING ACTIVITY OF A MITOCHROME PREPARATION

The pH of the incubation medium was 7.0.

Additions	*μatoms P exchanged/mg protein/h*
None	6.1
Mitochrome (0.41 mg protein)	0.4
Mitochrome (0.41 mg protein), extracted with iso-octane	4.0
Isooctane extract of mitochrome	0.4
Isooctane extract + 4 mg serum albumin	5.0

Development of uncoupling activity in cytochrome preparations

In the course of the preparation of mitochrome by the method of Polis and Shmukler[2], absorption bands of cytochrome *b* were observed with the microspectroscope. However, the bands disappeared when the solution was kept at 4° for a few days. As already described[4], purified preparations of cytochrome $(a + a_3)$ and of cytochrome *b*, obtained by the action of crude trypsin in the presence of cholate on a particulate preparation obtained from heart muscle, were transformed to mitochrome on standing for a few days at 4°.

Table V shows that during standing at 4°, there is an increase in the amount of uncoupling material which can be extracted from these cytochrome preparations.

Properties of the uncoupling agent

Tables VI and VII show that the material extractable from mitochrome with isooctane has all the properties of an uncoupling agent. It uncouples oxidative phosphorylation, stimulates ATPase and inhibits the P_i-ATP exchange (Table VI), and also decreases the respiratory-control index (Table VII). The respiratory-control index is the ratio between the rates of respiration in the presence of ADP (maintained by the addition of hexokinase and glucose), and in its absence. Table VII shows the

TABLE V

EXTRACTION OF UNCOUPLING LIPID FROM FRESH AND AGED CYTOCHROME PREPARATIONS

The preparations were extracted with isooctane, and the isooctane extracts added to the reaction medium (pH 7.0) for measurement of the exchange reaction.

Preparation extracted with isooctane	*Days at 4°*	*Exchange (µatoms P/mg protein/h)*
None	—	6.1
Cytochrome *a* + a_3	0	4.7
	7	2.1
	10	1.5
Cytochrome *b*	0	3.0
	5	0.9

TABLE VI

EFFECT OF ISOOCTANE EXTRACT OF A MITOCHROME PREPARATION ON OXIDATIVE PHOSPHORYLATION, ATPASE AND P_i-ATP EXCHANGE OF LIVER MITOCHONDRIA

Reaction	*Control*	*Isooctane extract of mitochrome added*[*]
Oxidative phosphorylation (P:O)[**]	1.45	0.01
ATPase (µatoms P/mg protein/h)[***]	0.3	4.0
P_i-ATP exchange (µatoms P/mg protein/h)[§]	4.1	0.4

[*] About 17 µg dry wt. (dissolved in 0.04 ml 96% ethanol).

[**] Succinate as substrate, pH 7.5.

[***] pH 6.5.

[§] pH 7.0.

TABLE VII

THE EFFECT OF THE ISOOCTANE EXTRACT OF MITOCHROME ON RESPIRATORY CONTROL OF LIVER MITOCHONDRIA

Glutamate substrate, pH 7.5.

Isooctane extract	Q_{O_2}		*Respiratory-control index*
	No hexokinase, Glucose	*+ Hexokinase, Glucose*	
0	11.0	52.0	4.7
Approx. 15 µg dry wt.	14.8	48.7	3.3
Approx. 30 µg dry wt.	22.0	43.4	2.0

decrease of this ratio brought about by the addition of the isooctane-extractable material, due partly to inhibition of the respiration in the presence of ADP, and partly to stimulation of the respiration in the absence of ADP.

The first indication of the chemical nature of the uncoupling material was obtained by the experiment shown in Table VIII. To the isooctane-extractable material, dissolved in 4 ml 96 % ethanol, was added 1 ml 5 *N* KOH and 25 ml water. The solution was extracted three times with 30 ml petroleum ether, and the combined petroleum ether extract, after washing with water, was evaporated to dryness and taken up in 4 ml 96 % ethanol. This solution represented the non-acidic fraction. The alkaline aqueous layer was acidified with HCl to pH 1–2 and extracted with petroleum ether as before. The final extract, dissolved in 4 ml 96 % ethanol, represented the acidic fraction. Table VIII shows that practically all the uncoupling activity resided in the acidic fraction. Similar results were obtained when the mitochrome preparation was extracted with ethanol.

TABLE VIII

DEMONSTRATION THAT UNCOUPLING ACTIVITY OF ISOOCTANE AND ETHANOL EXTRACTS OF MITOCHROME RESIDES IN THE ACID FRACTION

The additions given were all dissolved in 96 % ethanol. The final concentration of alcohol in the test medium was about 2 %. 1.03 mg mitochondrial protein used.

Additions	*ATPase (µatoms P/mg protein/h) at pH*						
	5.5	*6.0*	*6.5*	*7.0*	*7.5*	*8.0*	*8.5*
None	0.73	0.41	0.29	0.33	0.63	1.25	1.56
Isooctane extract	2.35	4.31	4.13	3.25	2.28	1.45	1.50
"Non-acid fraction"	1.15	0.88	0.55	0.62	0.93	1.82	1.71
"Acid fraction"	2.30	3.53	3.07	2.07	1.95	1.97	2.00
Ethanol extract	3.78	4.13	3.23	2.37	1.81	1.46	1.64
"Non-acid fraction" of the former	0.92	0.63	0.41	0.55	0.88	1.26	1.50
"Acid fraction" of the ethanol extract	3.89	4.61	3.34	2.41	1.38	1.57	1.96

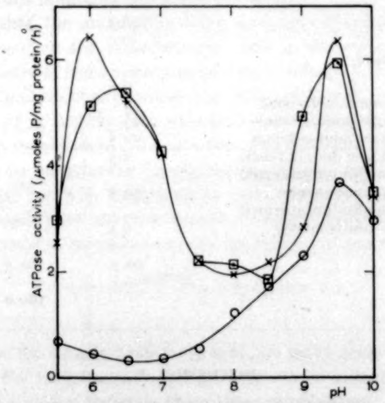

Fig. 2. The effect of the isooctane extract of mitochrome and of oleic acid on the ATPase activity of liver mitochondria. ○—○, 0.03 ml ethanol; ×—×, 0.03 ml ethanol containing approx. 30 µg (dry wt.) isooctane extract of mitochrome; ⊡—⊡, 0.03 ml ethanol containing approx. 20 µg oleic acid.

Fig. 2 shows that the pH-activity curves of the ATPase obtained by the addition of the uncoupling agent, and of about the same amount of oleic acid are quite similar. (The actual shape of the pH-activity curve depends upon the concentration of uncoupler; BORST AND LOOS, unpublished.)

Table IX shows an analysis, by gas chromatography, of the acidic fraction of an isooctane extract of mitochrome, after methylation with diazomethane. This analysis was kindly carried out and interpreted by Messrs. R. K. BEERTHUIS AND J. H. RECOURT of the Unilever Research Laboratory at Vlaardingen. It is noteworthy that 61.5 % of the acids are unsaturated, a value in close agreement with the 67 % found by BALL and his colleagues[15] for the fatty acids present in the lipids of a respiratory-chain preparation isolated from beef-heart muscle. The isooctane extract of mitochrome contains sufficient long-chain fatty acids to account for its uncoupling activity. Quantitative comparisons of the uncoupling activity of various long-chain fatty acids have been carried out by P. BORST AND J. A. LOOS and will be reported elsewhere.

TABLE IX

COMPOSITION OF ACID FRACTION OF ISOOCTANE EXTRACT OF MITOCHROME

Acid	*Content* *% (w/w) of total acid*	
Saturated		
C_{12}	1	
C_{14}	3.5	
C_{15}	trace	
C_{15}, branched	trace	
C_{16}	24	
C_{17}	trace	
C_{17}, branched	trace	
C_{18}	10	
	38.5	38.5
Unsaturated		
C_{14}	0.5	
C_{16}, one double bond	4	
C_{18}, one double bond	23	
C_{18}, two double bonds	10.5	
C_{18}, three double bonds	2.5	
C_{20}, highly unsaturated (4 components)	14.5	
C_{22}, highly unsaturated (2 components)	6.5	
	61.5	61.5
		100.0

DISCUSSION

The experiments reported in this paper make it clear that the uncoupling activity of mitochrome preparations resides in an uncoupling agent which can be extracted by organic solvents, rather than in the haem compound mitochrome. This uncoupling material binds firmly to albumin, which counteracts the uncoupling by both mitochrome preparations and iso-octane extracts. It is interesting that Polis and Shmukler[2] found that serum albumin bound the haemoprotein mitochrome so firmly that the two could not be separated electrophoretically. It seems likely that this binding between the two proteins occurs through the lipid uncoupling agent.

The experiment described in Table VIII establishes that the uncoupling lipid material is acidic in nature. Gas chromatography showed that the acidic fraction consisted of a mixture of saturated and unsaturated long-chain fatty acids, oleic acid[*] (23 %), linoleic acid[*] (10.5 %), linolenic acid[*] (2.5 %) and highly unsaturated long-chain acids (21 %) comprising 57 % of the weight. In studies which will be published elsewhere P. Borst and J. A. Loos have shown that oleic acid and linoleic acid are much more highly active as uncoupling agents than the corresponding saturated fatty acids, and, moreover, that there are qualitative differences in the form of the pH-activity curve obtained with oleic and stearic acids, that of oleic acid resembling the curve obtained with the lipid extracted from mitochrome (see Fig. 2). It seems very likely, therefore, that the uncoupling activity of mitochrome can be ascribed to the oleic acid, linoleic acid, linolenic acid and higher unsaturated fatty acids which it contains. It is known that serum albumin firmly binds long-chain saturated and unsaturated fatty acids, particularly oleic acid[16]. Davis and Dubos[17] showed that serum albumin protects erythrocytes against haemolysis by oleic acid.

These findings recall those of Pressman and Lardy[18] who isolated from microsomes a heat-stable, acetone-soluble fraction which stimulated the respiration of liver mitochondria in a medium deficient in phosphate acceptor, and stimulated the ATPase. The active substances were later identified as a mixture of long-chain fatty acids[19,20]. Pressman and Lardy[18] speculated on the possible regulatory role of the microsomal fatty acids on mitochondrial respiration, a possibility which would appear to be strengthened now that we have found similar acids in mitochondrial preparations. This point is further discussed elsewhere[21].

It seems likely that the uncoupling fatty acids are liberated by hydrolysis of a fatty acid ester present in the mitochondria, and in the cytochrome preparations which are probably derived from mitochondrial membranes. J. A. Loos (unpublished) has found that esterification of the uncoupling fatty acids with diazomethane brought about a loss of uncoupling activity. The mechanism of the liberation of the uncoupling fatty acids is not yet established. The fact that the transformation of isolated cytochrome preparations to mitochrome occurs simultaneously with the liberation of the uncoupling fatty acids could be explained in three ways: (a) the liberation of fatty acids from lipids, possibly an enzymic reaction, occurs independently of the transformation of cytochrome to mitochrome; (b) the following reaction takes place:

$$\begin{tikzcd}
\text{cytochrome} \arrow[r, "\text{ageing}"] \arrow[d, dash] & \text{mitochrome} + \text{L} \\
\text{L} &
\end{tikzcd}$$

where L is written for the unsaturated fatty acid; (c) fatty acids liberated from esters during ageing cause the transformation of cytochrome to mitochrome. Further work is required to enable a choice between these three possibilities.

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[*] The positions of the double bonds in the unsaturated C_{18} acids shown in Table IX were not determined. Therefore, a positive identification of these acids as oleic, linoleic and linolenic acids was not made.

Recent experiments[22] have provided evidence that the release of uncoupling fatty acids in mitochondrial preparations is an enzymic process. The uncoupling can be demonstrated by direct extraction with isooctane of a liver mitochondrial preparation after incubation with 0.001 *M* $CaCl_2$ for 90 min at 35°. The release was inhibited by 0.002 *M* EDTA, 0.002 *M* ATP and $5 \cdot 10^{-4}$ *M* diisopropylfluorophosphate, and prevented by previous boiling of the mitochondrial preparations, or by addition of trichloroacetic acid. Further studies on the nature of the enzyme involved are in progress.

The mechanism of the uncoupling activity of unsaturated fatty acids is not yet clear. It is unlikely to be due to disruption of the mitochondrial structure brought about by the surface activity of the unsaturated fatty acids since the uncoupling is reversible (P. Borst, unpublished).

It appears not unlikely that the stimulation of oxidative phosphorylation brought about by the addition of albumin to suspensions of mitochondria isolated from insect thoracic muscle[23–25] or from *Galleria* larvae[26] is also due to binding of uncoupling unsaturated fatty acids. This possibility is currently under examination in this laboratory.

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UNCOUPLING OF OXIDATIVE PHOSPHORYLATION AND INHIBITION OF ATP–P_i EXCHANGE BY A SUBSTANCE FROM INSECT MITOCHONDRIA

LECH WOJTCZAK AND ANNA B. WOJTCZAK

Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw (Poland)

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SUMMARY

Human and bovine serum or plasma albumins, and to a smaller degree β-lactoglobulin, stimulate oxidative phosphorylation and the ATP–P_i exchange reaction in mitochondria from larvae of the wax-moth, *Galleria mellonella* L. (Lepidoptera). This effect has been shown to be due to removal by these proteins of an uncoupling agent that is present in isolated insect mitochondria. It has been demonstrated that this uncoupling agent contains fatty acids, of which palmitic, stearic, oleic, linoleic and linolenic acids have been identified by paper chromatography.

INTRODUCTION

It is known that oxidative phosphorylation in isolated insect mitochondria is usually less efficient than in mitochondria from mammalian liver or heart. The yield of phosphorylation in insect tissue particles can be increased by the addition to the incubation medium of serum albumin[1,2] or of some other proteins[3]. This effect is best seen with sarcosomes from thoracic muscles of the housefly in which no phosphorylation is observed until bovine serum albumin is added[1]. An explanation has been proposed[1] according to which this stimulatory effect may be due to preservation of mitochondrial integrity by colloidal substances, as suggested by WATANABE AND WILLIAMS[4]. This explanation has recently appeared to be unsatisfactory since it has been shown[5] that, among a variety of proteins tested, only a limited number has the stimulatory effect on oxidative phosphorylation in housefly sarcosomes.

In a preliminary communication[6] we described a stimulatory effect of serum albumin on oxidative phosphorylation and the ATP–P_i exchange reaction in mitochondria from wax-moth larvae and were able to show that this effect is due to the binding of albumin with an uncoupling agent. In this paper more details of these experiments are reported and evidence is presented that the uncoupling substance contains a mixture of fatty acids.

Abbreviations: P_i, orthophosphate; AMP, adenosine 5′-phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; pCMB, *p*-chloromercuribenzoate.

MATERIALS AND METHODS

Rat liver mitochondria were isolated in 0.25 *M* sucrose as described by Myers and Slater[7]. Wax-moth mitochondria were obtained from whole fully grown larvae of the wax-moth, *Galleria mellonella* L. by the same procedure: mitochondria used in experiments on ATP–P_i exchange were isolated and suspended in 0.25 *M* sucrose; mitochondria used for measurements of phosphorylation were isolated in 0.25 *M* sucrose containing 5 m*M* ATP and were finally suspended in 0.25 *M* sucrose containing 1 m*M* ATP, as recommended by Holton *et al.*[8] for heart sarcosomes.

Oxidative phosphorylation was investigated in a medium containing: KCl, 20 µmoles; $MgCl_2$, 6 µmoles; EDTA, 2 µmoles; phosphate buffer, pH 7.2, 10 µmoles; Tris–HCl buffer, pH 7.2, 18 µmoles; AMP, 0.5 µmole; ATP, 0.2 µmole; sodium succinate or glutamate, 8 µmoles; glucose, 10 µmoles; sucrose, 50 µmoles; hexokinase, 200 Kunitz–McDonald[9] units; mitochondria containing 2–4 mg protein. Final volume was 1.0 ml; temperature, 25°; incubation time, 20–40 min. Oxygen uptake was measured in micro-flasks (about 5 ml capacity) of a conventional Warburg apparatus; phosphorylation was determined by measuring the amount of either hexose monophosphate formed[10] or orthophosphate disappeared[11] (both values appeared to be consistent within the limits of experimental error).

ATPase activity was determined as described by Myers and Slater[7] in a medium containing: KCl, 112.5 µmoles; $MgCl_2$, 4.5 µmoles; EDTA, 1.5 µmoles; ATP, 3 µmoles; sucrose, 162.5 µmoles; mitochondria containing about 1 mg protein. Total volume was 1.5 ml; pH 7.4; incubation time, 15 min; temperature, 20°.

ATP–P_i exchange was investigated using a slightly modified incubation medium of Hülsmann *et al.*[12] which contained in a total volume of 1.0 ml: KCl, 100 µmoles; $MgCl_2$, 4 µmoles; EDTA, 1.5 µmoles; Tris–HCl buffer, pH 7.5, 34 µmoles; phosphate buffer, pH 7.5, containing about 10^6 counts/min ^{32}P, 3 µmoles; ATP, 5 µmoles; sucrose, 12.5 µmoles; mitochondria containing about 1 mg protein. Incubation time was 15 min at about 20°. [^{32}P]ATP formed was determined as described by Nielsen and Lehninger[13]. The values expressed as µatoms P exchanged/mg mitochondrial protein/h are corrected for ATPase activity, assuming that the rates of exchange reaction and of hydrolysis of ATP are linear within the time of experiment.

Preliminary experiments on ATP–ADP exchange were carried out with the same incubation medium as for ATP–P_i exchange except that $^{32}P_i$ was omitted and 3 µmoles ADP containing about 150,000 counts/min [^{32}P]ADP were added. The medium contained 0.01 *M* NaF. Incubation time was 5 min at 20°. The nucleotides were separated on a Dowex-2 column and the radioactivity was measured with a thin-window Geiger–Müller counter.

Protein content in mitochondrial suspensions was determined by the biuret method according to the procedure of Cleland and Slater[14].

Chromatography of fatty acids was carried out on Whatman No. 3 filter paper impregnated with liquid paraffin[15]. Spots of fatty acids were made visible by the copper acetate–potassium ferricyanide procedure and those of unsaturated fatty acids by alkaline permanganate treatment as described by Kaufmann[15].

AMP and ADP were products of Light & Co. Ltd.; ATP was obtained from Fluka AG or was prepared from rabbit muscles[16]. Protein preparations were obtained as follows: bovine plasma albumin fraction V and protamine sulfate, from Light & Co.

Ltd.; crystalline bovine serum albumin, bovine β-lactoglobulin and cytochrome *c*, from Nutritional Biochemicals Corporation; human serum albumin fraction V, human γ-globulin and human haemoglobin, from the Institute of Haematology in Warsaw. Anserine and carnosine were kindly offered by Prof. S. E. SEVERIN, Moscow. Hexokinase (crude, type II) was obtained from Sigma Co.

[^{32}P]ADP was prepared as described by COLOWICK AND KAPLAN[17].

RESULTS

Effect of proteins on oxidative phosphorylation and on the ATP–P_i exchange reaction in wax-moth mitochondria

Values of the P:O ratio as low as 0.2 were obtained with succinate if no protein was added to the incubation medium. With serum or plasma albumin, however, the efficiency of phosphorylation was increased considerably and P:O ratios as high as 1.4 were obtained. Among a number of proteins tested (Table I) only β-lactoglobulin was found to have a similar although smaller effect.

Addition of human or bovine serum albumin not only strongly stimulated the esterification of inorganic phosphate, but also increased O_2 uptake by the respiring mitochondria; mean values from 10 experiments were: 2.80 μatoms O_2/mg mitochondrial protein/h if no albumin was added and 3.78 μatoms O_2/mg protein/h in the presence of 9 mg albumin.

Further experiments showed that serum and plasma albumins and β-lactoglobulin but none of the other proteins tested appreciably increased the ATP–Pl exchange catalysed by wax-moth mitochondria (Table I).

Fig. 1 illustrates the effect of various amounts of serum albumin on the P:O ratio and ATP–P_i exchange. It can be seen that as little as 1 mg albumin/ml incubation medium produces half maximum stimulation of the exchange reaction, whereas higher concentrations of albumin are necessary to increase the net phosphorylation.

TABLE I

EFFECT OF PROTEINS ON PHOSPHORYLATION COUPLED TO OXIDATION OF SUCCINATE AND ON ATP–P_i EXCHANGE IN MITOCHONDRIA FROM WAX-MOTH LARVAE

*Protein added**	*Oxidative phosphorylation (P:O)*	*Exchange reaction (μatoms P/mg mitochondrial protein/h)*
None	0.2 (10)	0.1 (10)
Crystalline bovine serum albumin	1.1 (5)	1.1
Bovine plasma albumin, fraction V	1.3 (5)	2.2 (8)
Human serum albumin, fraction V	1.4	1.9 (3)
Egg albumin	0.1	0.1
Human serum γ-globulin	0.3	0.2
β-Lactoglobulin (bovine)	0.4 (2)	2.1 (2)
Haemoglobin (human)	0.1	0.1 (2)
Cytochrome *c*		0.1
Casein	0.0	0.0
Insulin	0.1	0.2
Protamine sulfate		0.1

Number of experiments, if more than one, is indicated in parentheses.

* 9.0 mg were added in phosphorylation tests and 4.5 mg in ATP–P_i exchange experiments.

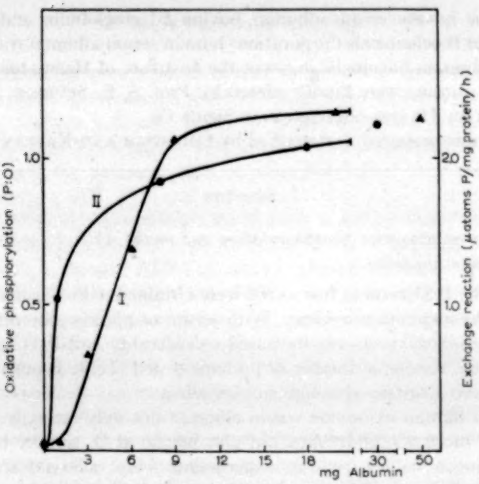

Fig. 1. Effect of various quantities of bovine plasma albumin on oxidative phosphorylation (Curve I) and ATP–P_i exchange (Curve II) in wax-moth mitochondria (substrate: succinate)

The stimulatory effect on the exchange reaction was not observed with any of the following amino acids tested separately: glycine, alanine, serine, cysteine, phenylalanine, tyrosine, tryptophan, histidine, methionine, valine, leucine, arginine, lysine, aspartic acid, glutamic acid*, threonine and proline (20 μmoles of each), nor with the peptides: glutathione (20 μmoles), carnosine (10 μmoles) and anserine (10 μmoles).

Sacktor *et al.*[5] have suggested that sulphhydryl groups of albumin are involved in the stimulatory effect on phosphorylation. This suggestion was based on the observation that albumin treated with pCMB had no stimulatory effect. In our experiments also, pCMB-treated albumin did not stimulate the exchange reaction. This was, however, due rather to an inhibition of the exchange reaction by pCMB bound with albumin than to a lack of stimulation since native and pCMB-treated albumins added together also did not increase the rate of the exchange reaction. Moreover, the iodination of albumin, which is known to oxidize sulphhydryl groups, did not appreciably diminish the stimulatory properties. All this suggests that free SH groups of albumin are not necessarily involved in the stimulation of ATP–P_i exchange in insect mitochondria.

ATPase activity

Wax-moth mitochondria contain a Mg-activated ATPase (on an average 3 μmoles ATP hydrolysed/mg mitochondrial protein/h) which is not further activated by 2,4-dinitrophenol. Serum albumin (4.5 mg/ml incubation medium) usually diminished this activity by a half. This effect of added albumin on ATPase activity was not always observed, however, which is in sharp contrast with the considerable and reproducible effect on the ATP–P_i exchange reaction and on the net phosphorylation.

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* 10^{-3} *M* KCN was present in the incubation medium, in this case to inhibit the oxidation of glutamate and to prevent the net phosphorylation which is coupled with that process.

"Activation" of wax-moth mitochondria and isolation of an inhibitor of ATP–P_i exchange

Further experiments showed that washing wax-moth mitochondria with an albumin–sucrose solution was almost as effective in stimulating ATP–P_i exchange as was the addition of albumin to the reaction medium. In this procedure isolated mitochondria were suspended in 0.25 M sucrose containing 3 % bovine or human serum albumin and centrifuged. They were washed subsequently with 0.25 M sucrose to remove the remaining albumin, centrifuged and resuspended in 0.25 M sucrose. Mitochondria which had been "activated" in this way catalysed on an average the exchange of 1.1 μatoms P/mg mitochondrial protein/h, which is approximately half of the average exchange in the presence of 4.5 mg albumin in the incubation medium, but is many times greater than the exchange by "non-activated" mitochondria in the absence of albumin (*cf.* Table I). This state of "activation" was, however, transitory; the ability to catalyse the exchange reaction in the absence of albumin decreased on standing in sucrose solution at 0° and after storage for 1 h no "activation" could be observed.

Washing the mitochondria with a sucrose–albumin solution was also effective in promoting oxidative phosphorylation, although the P:O ratio was still rather low.

The sucrose–albumin solution that had been used to wash the mitochondria (henceforth termed "washing fluid") was heated for a few minutes at 100° in order to coagulate the albumin, and a portion of the suspension obtained was added to the incubation medium which contained "activated" wax-moth mitochondria. This resulted in a considerable decrease of ATP–P_i exchange (Table II, Expt. 1). A similar inhibitory effect was also produced by the precipitate of coagulated albumin from the washing fluid but not by the clear supernatant obtained by centrifuging off the precipitated albumin. A control test showed that pure serum albumin if thermally coagulated had only a slight inhibitory effect.

TABLE II

INHIBITION OF ATP–P_i EXCHANGE IN "ACTIVATED" WAX-MOTH MITOCHONDRIA

Expt.	*Addition*	*Exchange (μatoms P/mg protein/h)*	*Inhibition %*
1	None	0.80	
	"Washing fluid" (heated)	0.39	51
	Coagulated albumin from the washing fluid	0.22	73
	Albumin-free supernatant from the washing fluid	0.76	5
	Bovine serum albumin (thermally coagulated)	0.67	16
2	None	0.67	
	Ethanol extract	0.05	92
	Benzene extract	0.12	82
	Ethanol-extracted albumin from the washing fluid	0.65	3

In another experiment portions of the coagulated albumin from the washing fluid were extracted with organic solvents: ethanol, benzene and acetone. The extracts were placed in incubation tubes and the solvents were evaporated prior to the addition of incubation medium and mitochondria. It appeared (Table II, Expt. 2) that the extracts strongly inhibited the exchange reaction in "activated" mitochondria, whereas the albumin after extraction was without inhibitory effect.

On the basis of these results, the following routine procedure was adopted to isolate the inhibitory substance. Mitochondria from 50 larvae (about 70 mg mitochondrial protein) were suspended in 0.25 *M* sucrose and an equal volume of 0.25 *M* sucrose containing 6 % serum albumin was added. The mitochondrial suspension was then allowed to stand for 30–60 min at 0° and centrifuged during 10 min at 12,500 × *g* in order to allow the mitochondria to settle. The supernatant fluid ("washing fluid") was decanted and heated for 5 min at 100°. It was then centrifuged and the precipitate of coagulated albumin was washed once with water and afterwards extracted with two portions of 1.5 ml ethanol. This ethanol extract contained the inhibitor of the ATP–P_i exchange reaction; 0.3 ml of the extract was sufficient for complete inhibition of the exchange reaction with "activated" mitochondria in a usual test system.

Effect of the inhibitory substance on rat liver mitochondria

The substance obtained as described above appeared to inhibit the ATP–P_i exchange reaction not only in "activated" wax-moth mitochondria but also in rat liver mitochondria. In addition, it uncoupled oxidative phosphorylation and stimulated the latent ATPase of liver mitochondria, as is shown in Table III. All these effects could be abolished by the addition of serum albumin.

TABLE III

EFFECT OF ETHANOL EXTRACT FROM THE WASHING FLUID ON PHOSPHORYLATION COUPLED TO THE OXIDATION OF GLUTAMATE, ATP–P_i EXCHANGE AND ATPASE ACTIVITY IN RAT-LIVER MITOCHONDRIA

Additions	*Oxidative phosphorylation (P:O)*	*Exchange reaction (µatoms P/mg protein/h)*	*ATPase activity (µmoles/mg protein/h)*
None	2.5	2.3	0.2
Ethanol extract, 0.5 ml	0.1	0.1	2.0
Ethanol extract, 0.5 ml and serum albumin, 9.0 mg	2.8	1.7	0.0

Ethanol was evaporated prior to the addition of incubation medium and mitochondria.

Preliminary experiments were made to investigate a possible effect of this substance on ATP–ADP exchange. This reaction may proceed not only as a result of mechanisms related to oxidative phosphorylation[18] but also as result of some other enzymic processes. In our experiments the activity of adenylate kinase was partly inhibited when fluoride was included in the medium. It was shown in a separate experiment that the remaining activity of adenylate kinase was responsible for not more than 10 % of the exchange observed. Participation of other mechanisms that might be expected to convert labeled ADP to ATP (*cf.* WADKINS AND LEHNINGER[18]), however, could not be excluded in the present investigations. The results have therefore only a tentative character.

The ATP–ADP exchange reaction in rat liver mitochondria appeared to be several times more intense than the ATP–P_i exchange reaction and, in contrast to the latter, was not inhibited by the ethanol extract obtained as described in the preceding section. ATP–ADP exchange in wax-moth mitochondria was almost equally intense in the absence as in the presence of serum albumin.

Chemical characterization of the inhibitor of the ATP–P_i exchange reaction

The solubility in organic solvents suggested that the inhibitory substance was of a lipid character. Preliminary experiments showed that the inhibitor was not destroyed by heating in 1 *N* HCl or 1 *N* KOH for 3 h at 100°. This suggested that it was not fat, or phospholipid or other hydrolysable compound. A portion of the ethanol extract containing the inhibitor was made alkaline with 6 *N* KOH and, after dilution with water, extracted with ether (extract I). The water phase was acidified with HCl and again extracted with ether (extract II). Extract I, which could contain fats, phospholipids and unsaponifiable material, was without effect on the ATP–P_i exchange reaction in rat liver mitochondria, while extract II was strongly inhibitory. This extract might contain fatty acids.

A small drop of an ethanol solution of the inhibitor placed on a piece of filter paper gave positive colour tests for fatty acids in the copper acetate–potassium ferricyanide procedure and for unsaturated fatty acids with alkaline permanganate solution. On chromatography in 93 % acetic acid, one spot was detected by copper acetate–potassium ferricyanide treatment that corresponded to palmitic or/and oleic acids. Occasionally another spot corresponding to stearic acid could be observed with more concentrated extracts of the inhibitor. With an alkaline permanganate solution three spots were distinctly visible. The most proximate to the starting line, with the same R_F value as the spot found with copper acetate–potassium ferricyanide, corresponded to oleic acid; the two remaining spots had R_F values of linoleic and linolenic acids. These acids were probably present in far smaller quantities than oleic acid and could not be detected by the copper acetate–potassium ferricyanide procedure for fatty acids, which is less sensitive than the permanganate reagent for unsaturated bonds. In order to ascertain whether palmitic acid was also present, the inhibitor was chromatographed in acetic acid containing hydrogen peroxide*. Under these conditions unsaturated fatty acids are destroyed. Indeed, if a sufficient quantity of the inhibitor was present a distinct spot of palmitic acid and a faint one of stearic acid could be observed.

It is known[19] that preparations of serum albumin usually contain small quantities of bound fatty acids. We have demonstrated, however, that the original content of fatty acids in the amount of albumin used in washing the mitochondria is too small to be detected chromatographically by the procedure adopted here.

An approximate evaluation of the amount of oleic acid present in the inhibitor was done by comparing the intensity of spots on chromatograms with spots given by known quantities of the acid. It appeared that about 0.7 mg of oleic acid could be obtained from mitochondria from 50 larvae by the procedure described above.

To make sure that the inhibitory effect was due to fatty acids the inhibitor was chromatographed, various parts of chromatogram were eluted with ether and the

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* According to Dr. H. K. MANGOLD, Hormel Institute, Austin, Minnesota; private information from Prof. W. NIEMIERKO.

TABLE IV

INHIBITION OF ATP–P_i EXCHANGE IN RAT LIVER MITOCHONDRIA BY ETHER ELUATES FROM CHROMATOGRAMS OF THE INHIBITING SUBSTANCE (MEAN VALUES FROM 5 EXPERIMENTS)

Part of chromatogram eluted	*Inhibition* %
Origin	4
Spots of fatty acids	59
Solvent front	43

Ether was evaporated prior to the addition of reaction mixture and mitochondria.

eluates tested for inhibition of ATP–P_i exchange in rat liver mitochondria. It appeared (Table IV) that the eluates from the area containing fatty acids as well as from an area near the solvent front line produced a distinct inhibition, whereas the eluates from other parts of the chromatogram were without effect. It was also shown in a separate experiment that chemically pure salts of fatty acids inhibited ATP–P_i exchange in rat liver mitochondria: 100 μg of K oleate were sufficient to block the exchange almost completely and 100 μg of Na stearate diminished the rate of the exchange reaction by half.

DISCUSSION

Serum albumin is without effect on oxidative phosphorylation in fresh mammalian liver mitochondria but, a pronounced protective effect has been observed on phosphorylation in "aged" mitochondria[20, 21]. This effect has been attributed[22] to the binding by albumin of an uncoupling substance, a haemoprotein called "mitochrome", which is liberated from mitochondria during ageing. The active component of mitochrome has been shown[12] to be extractable with organic solvents and has been recently identified[23] as a mixture of fatty acids.

The present investigation shows that the effect of serum albumin on oxidative phosphorylation and ATP–P_i exchange in wax-moth mitochondria is also due to the binding by albumin of an uncoupling substance which, in contrast to liver mitochondria, is present even in freshly prepared insect particles. This uncoupling agent can be isolated by washing wax-moth mitochondria with a sucrose–albumin solution and subsequent extraction of the washing fluid with organic solvents. The extract has been shown to contain fatty acids among which palmitic, stearic, linoleic and linolenic have been identified. It is thus evident that the composition of the uncoupling agent from insect mitochondria is similar to the composition of the lipid fraction of mitochrome as investigated by HÜLSMANN[23].

It is probable that besides the five fatty acids mentioned other active components are also present in the uncoupling substance from wax-moth mitochondria. This is indicated by inhibition of ATP–P_i exchange produced by eluates from paper chromatograms of the inhibitor.

An explanation that the effect of some proteins on oxidative phosphorylation in insect mitochondria may be due to the binding of an uncoupling substance has been proposed some years ago by REES[3] and has been supported recently by SACKTOR[5], although no clear-cut experimental evidence has been given so far. Recently, AVI-DOR

AND GONDA[24] have demonstrated a stimulatory effect of serum albumin on the ATP–P_i exchange reaction in tissue particles from mosquitoes and house flies. This effect is produced if albumin is present in either the reaction medium or in the washing solution. This is very consistent with our previous report[6] and with the present results with mitochondria from wax-moth larvae.

The fact that, among a variety of proteins investigated, serum albumin and β-lactoglobulin are most effective in promoting oxidative phosphorylation and stimulating the ATP–P_i exchange reaction can be understood on the basis that these two proteins have a very high binding property towards anions (*cf.* KLOTZ[25]) and particularly towards fatty acids (*cf.* DEUEL[26]).

There is no clear evidence why the isolation procedure that gives extensively phosphorylating mitochondria from mammalian liver or heart is usually unsuitable for isolation of phosphorylating particles from insects. One can suspect that some lipid constituents which give rise to free fatty acids are more labile in mitochondria from insects than from mammalian tissues or that enzymes are present in insect tissues which split mitochondrial lipids, with the liberation of free fatty acids. This question needs to be investigated further.

It is worth while mentioning that oxidative phosphorylation in particles from pigeon skeletal muscles[27], from rat brain[28] and from *Azotobacter vinelandii*[29] is also stimulated or protected by an addition of serum albumin. It may be supposed that the mechanism of this effect is similar to that acting in the case of insect mitochondria.

Uncoupling of oxidative phosphorylation and stimulation of latent ATPase by fatty acids have already been demonstrated by PRESSMAN AND LARDY[30, 31] with rat liver mitochondria. The present investigation shows, in addition, that fatty acids are inhibitory in ATP–P_i exchange, but probably not in ATP–ADP exchange reactions. The mechanism of these effects is not clear. It can be supposed that fatty acids may damage mitochondrial structure as surface-active agents[31], thus uncoupling oxidative phosphorylation and stimulating latent ATPase, or that they have a more specific inhibitory effect on some enzymic processes involved in the mechanism of oxidative phosphorylation.

The following equations have been formulated[18] for ATP synthesis coupled with electron transport:

$$\text{Carrier} + X \underset{}{\overset{\text{electron transfer}}{\rightleftarrows}} \text{Carrier} \sim X \tag{1}$$

$$\text{Carrier} \sim X + P_i \rightleftarrows \text{Carrier} + X \sim P \tag{2}$$

$$X \sim P + ADP \rightleftarrows X + ATP \tag{3}$$

Reactions (2) and (3) are involved in ATP–P_i exchange, reaction (3) in ATP–ADP exchange. Reactions (3) and (2) followed by an irreversible hydrolysis of Carrier ~ X:

$$\text{Carrier} \sim X \xrightarrow{+H_2O} \text{Carrier} + X \tag{4}$$

are the basis of mitochondrial ATPase activity. The effects of fatty acids on oxidative phosphorylation, mitochondrial ATPase and ATP–P_i exchange may be explained by the assumption[23] that fatty acids, like other uncoupling agents, promote the irreversible reaction (4). Assuming that the rate of reaction (3) is far greater than that of reaction (2), no considerable inhibitory effect on ATP–ADP exchange would be expected—which is indeed the case.

It was observed in the present investigation that serum albumin increased the rate of substrate oxidation by wax-moth mitochondria in the presence of phosphate acceptor. Similar results have been obtained[1,2,5,32] with mitochondria from other insects. This effect can also be accounted for on the basis of removal of fatty acids by added albumin. As assumed by Hülsmann[23], fatty acids not only promote splitting of the compound Carrier $\sim X$ but also bind with free Carrier, thus lowering the "steady-state" concentration of the Carrier which is necessary for electron transfer (reaction (1)).

ACKNOWLEDGEMENTS

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BACILLUS SUBTILIS α-AMYLASE, A ZINC-PROTEIN COMPLEX*

ERIC A. STEIN AND EDMOND H. FISCHER

Department of Biochemistry, School of Medicine, University of Washington, Seattle, Wash. (U.S.A.)

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SUMMARY

1. Crystalline *Bacillus subtilis* α-amylase, which has been reported to be a calcium metalloenzyme, also contains small amounts of zinc. Under normal conditions, this enzyme exists in the form of a dimer.

2. Dissociation of the dimer takes place in the presence of metal-binding agents whereas dimerization is induced by zinc ions. One atom of zinc per molecule of dimer is taken up by the protein during dimerization and released as dissociation proceeds according to the following scheme:

$$[\mathrm{Prot} - \mathrm{Ca}_x] - \mathrm{Zn} - [\mathrm{Prot} - \mathrm{Ca}_x] \xrightleftharpoons[\mathrm{Zn}^{++}]{\text{Metal binding agents}} 2[\mathrm{Prot} - \mathrm{Ca}_x] + \mathrm{Zn}$$

3. Formation of this zinc–protein complex appears to be peculiar to *B. subtilis* α-amylase. Intermolecular cross-linking under the influence of zinc has not been observed with α-amylases from human saliva, hog pancreas, or *Aspergillus oryzae*.

4. Calcium ions do not induce dimerization of bacterial amylase.

INTRODUCTION

In the course of a comparative study of the structure of α-amylases from mammalian, bacterial, and fungal origin, it was established that these enzymes are resistant to proteolysis when combined with calcium ions. However, in the presence of metal-binding agents, α-amylases become exceedingly susceptible to proteolytic degradation[2,3], and the breakdown that ensues when sequestering agents are added to amylase preparations contaminated by traces of proteases is easily recognizable by ultracentrifugal analysis. In the absence of proteases, the behavior of α-amylases from human saliva, hog pancreas, and *Aspergillus oryzae* in the centrifugal field is no longer affected by treatment of the enzymes with chelating agents. By contrast, when bacterial amylase is subjected to a similar treatment, a significant decrease in the sedimentation velocity is observed. This anomalous behavior has prompted the study of the molecular transformations induced by sequestering agents in protease-free preparations of *Bacillus subtilis* α-amylase.

The following abbreviations are used: GP, Na-glycerophosphate; EDTA, Na-ethylenediamine tetraacetate.

* A preliminary report of this investigation was presented before the Forty-Eighth Annual Meeting of the American Society of Biological Chemists in Chicago, April 15–19, 1957 (1).

EXPERIMENTAL

Unless otherwise stated, *B. subtilis* α-amylase was purified from a bacterial concentrate ("H.T.") purchased from the Takamine Laboratory, Clifton, New Jersey, recrystallized two or three times and freed from proteolytic contaminants according to procedures described previously[2,4,5]. In a few instances the crystalline amylase from *B. subtilis* was prepared from another bacterial concentrate ("Biolase"), purchased from Kalle and Co., Wiesbaden, Germany[6]. Such preparations are referred to as "Biolase" in the text. GP was purchased from Eastman Organic Chemicals Department, Division of the Eastman Kodak Co. EDTA (Baker Chemical Co.) and all other chemicals were reagent grade. Radioactive ^{65}Zn was obtained in the form of a solution of $ZnCl_2$ in HCl from the Oak Ridge National Laboratory. The analysis supplied with the sample was 155 mC/g and 102.0 mg Zn/ml.

All incubations, dialyses, and ultracentrifuge runs were performed in the cold (2 to 10°). Except when otherwise indicated, viscosity measurements and ultracentrifugal analyses were performed on 0.5 to 0.7% protein solutions which were dialyzed overnight *vs.* large amounts (*ca.* 1000 volumes) of 0.1 *M* NaCl + 0.02 *M* GP–HCl buffer pH 7.0, using a rotating dialyzer equipped with cellulose casings (Visking Corporation, Chicago, Ill.). Sedimentation constant values were not extrapolated to zero protein concentration since it had been shown previously[6] that the sedimentation velocity of this enzyme is little influenced by changes in protein concentration.

The concentration and enzymic activity of amylase were measured as described previously[2]. Specific activities were determined before addition of sequestering agents, metal ions, etc., and after ultracentrifugal analysis. Since losses were usually lower than 10%, it can be assumed that little or no irreversible denaturation took place during the experiments described in this work.

Viscosity measurements were made in an Ostwald viscosimeter, with a time of flow for water at 4° of approximately 200 sec. The average of six determinations, which agreed within 0.2 sec, was used.

Sedimentation velocity measurements were made in the Spinco Model E ultracentrifuge equipped with a "Rotor Temperature Indicator and Control" device. All runs were made at a speed setting of 59,780 rev./min, and the actual average angular velocity was computed from odometer readings at the beginning and end of the period at full speed. Standard Dural cells of 12 mm with a 4° sector were consistently used.

Since the purpose of this work was not to determine the absolute value of the sedimentation constant of bacterial amylase, and since the sedimentation constants were used purely as an index of *relative* size, the highest degree of accuracy could be dispensed with. Thus, the duration of the ultracentrifuge runs was usually limited to 50 min at full speed during which time six exposures were taken at 8-min intervals. The sedimentation constants, S, were calculated from the equation:

$$\ln x = \omega^2 St + C$$

where x is the radial distance fron the center of rotation and ω is the angular velocity. The slope of the line of best fit was computed by means of the formula reported by ASKOVITZ[7]:

$$\text{mean rate of change} = \frac{\Sigma(2i - n - 1)x_i}{\Sigma(2i - n - 1)t_i} \qquad (i = 1,2, \ldots n)$$

This relation is particularly useful in the instance of an even number, n, of measurements and can be further simplified when the observations are made at regular time intervals Δt:

$$\text{mean rate of change} = \frac{\Sigma(2i - n - 1)x_i}{\Sigma(2i - n - 1)\Delta t/2}$$

The value thus obtained also represents the slope of the line of best fit provided by the more cumbersome method of least squares[7].

The error in the measurements of S was estimated to be well within 0.1 Svedberg unit, except for markedly polydisperse solutions where the error was somewhat larger. The sedimentation constants were corrected to 20°, using the factor $(\eta_t/\eta_{20})_w$. The temperature of the run was determined by averaging the temperature readings made at each exposure time. The S_{20} values were not corrected for the increment in relative viscosity due to the various buffers and salts used, since (a) this increment did not exceed 0.025 ± 0.015 at the average temperature of the runs, and (b) the same buffers were used both in the reference and in the test systems. The increase in relative viscosity caused by the addition of salts of divalent metals or sequestering agents to the test system only, did not exceed 0.02. The effect of this latter viscosity increment on the sedimentation constant would be of the order of 0.1 S. It could be safely neglected since the observed changes amounted to 1.5 to 2 S.

Radioactivity determinations were made on 1-ml samples in a well counter (Decimal Scaler Model 2105, Berkeley Scientific Corporation, Richmond, Cal.). To establish the calibration curve, the original radioactive zinc solution was diluted to concentrations ranging from 0.01 to 0.4 μg zinc (corresponding to approximately 300–12,000 counts/min) in 0.1 M acetic acid*, and a linear relationship was obtained. The counting efficiency was of the order of 10%. In all instances, the counting time was such that the statistical errors were kept below 2%[8]; the radioactivity measured was at least ten times above background.

RESULTS

Effect of sequestering agents on the sedimentation velocity of amylase

Preliminary experiments, a few of which are described in Table I, have indicated that at pH 7.0 recrystallized *B. subtilis* α-amylase gives a single, well-defined, and essentially symmetrical, peak (see Fig. 1a) in the ultracentrifuge, with a sedimentation constant of approximately 6 S. Saturation of the protein with Ca^{++} ions has no effect on the behavior of the enzyme in the ultracentrifuge. This is of interest since *B. subtilis* α-amylase binds from 3 to 7 or more gram-atoms Ca per mole, depending on the previous history of the material under investigation[3]. By contrast, the enzyme sediments with a strikingly lower velocity in the presence of chelating agents. Under a variety of conditions the sedimentation constant S_{20} is reduced by EDTA from approximately 6 to nearly 4 S. The enzyme in the S-4 form** is also homogeneous, as illustrated in Fig. 1b. When the ultracentrifugal analyses are performed in the alkaline range of pH, sedimentation constants slightly lower than 6 S are observed

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* Dilution in water gave erratic results, probably due to an absorption of Zn hydrate on glass surfaces during pipetting.

** The fast and slow sedimenting forms of bacterial amylase will be designated as S-6 and S-4 respectively.

TABLE I

EFFECT OF Ca^{++} IONS AND OF EDTA ON THE SEDIMENTATION CONSTANT OF *B. subtilis* α-AMYLASE

Run. No.	*Buffers*	*Additives*	*pH*	*Temperature*	*Protein concentration %*	S_{20}
1151	0.1 *M* NaCl + 0.02 *M* GP	—	7.0	4.0	0.5	6.0
1063	0.1 *M* NaCl + 0.02 *M* GP	—	7.0	5.0	0.6	6.0
1065	0.1 *M* NaCl + 0.02 *M* GP	0.01 *M* Ca-Acetate	7.0	5.5	0.5	6.0
1064	0.1 *M* NaCl + 0.02 *M* GP	0.01 *M* EDTA	7.0	3.5	0.5	4.1
1191	0.1 *M* NaCl + 0.02 *M* Na-Acetate	—	7.0	3.7	0.6	6.2
1194	0.1 *M* NaCl + 0.02 *M* Na-Acetate	0.01 *M* EDTA	7.0	4.5	0.5	4.3
1056	0.07 *M* GP	—	8.0	12.0	0.7	5.4
1058	0.07 *M* GP	0.005 *M* Ca-Acetate	8.0	8.5	0.6	5.4
1059	0.07 *M* GP	0.005 *M* EDTA	7.7	5.5	0.6	4.0
825	0.1 *M* Veronal-Acetate	—	8.55	20.0	0.7	5.3
864	0.1 *M* Veronal-Acetate	0.01 *M* EDTA	8.55	20.0	0.7	4.1

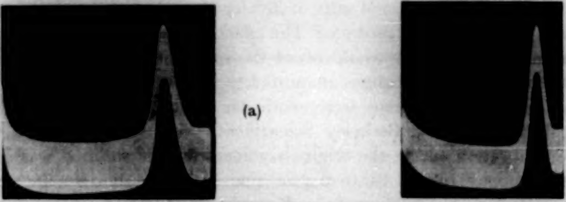

Fig. 1. Sedimentation pattern of bacterial amylase. The exposures were taken 50 min after the rotor had reached full speed (59,780 rev./min). Bar angle: 45°. Direction of sedimentation: from right to left. (a) Enzyme in the S-6 form (run No. 1063, Table I). (b) Enzyme in the S-4 form (run No. 1064, Table I).

even in the absence of chelating agents. These lower values are probably due to the metal-binding properties displayed by the buffers themselves under these conditions (*cf.* runs No. 1056, 1058, and 825, Table I).

To determine the influence of time on the transformations induced by EDTA, a 0.55 % amylase solution was first equilibrated in an 0.1 *M* NaCl–0.02 *M* GP buffer pH 7.0, and then 0.01 *M* EDTA was added. Aliquot samples were withdrawn at various intervals of time and analyzed in the ultracentrifuge. Results show that the S-6 to S-4 transformation reaches completion before the first exposure can be made (*i.e.*, 30 min after addition of EDTA). From there on, the S_{20} remains constant at a value of 4.1 to 4.2 for a period of several days. Removal of the free chelating agent by prolonged dialysis or neutralization of the sequestering agent by addition of Ca^{++} ions did not result in a reconversion of the S-4 to the S-6 form.

A variety of sequestering agents besides EDTA can be used to lower the sedimentation constant of bacterial amylase. Orthophenanthroline and 8-hydroxyquinoline appear to be as efficient as EDTA in bringing about the S-6 to S-4 conversion. However, when the enzyme is incubated with weaker metal-binding agents such as citrate or oxalate at the same concentration (0.01 *M*), heterogeneous sedimentation patterns or intermediate S_{20} values may be obtained, indicating that the S-6 to S-4 transformation does not reach completion. In this instance ***dialysis*** against the metal-binding agent may suffice to carry the reaction to completion. For instance, incubation of a 0.6 % amylase solution in 0.01 M Na-citrate and 0.1 M NaCl for 22 h at 5°, pH 7.0, resulted in a lowering of the S_{20} value from 5.9 to 4.7 only, whereas dialysis under the same conditions decreased the S_{20} value to 4.1 Treatment with a cation-exchanger, such as Amberlite IR-120, has also been used successfully to convert the enzyme to the S-4 form.

Viscosity studies

Viscosity determinations were made in order to establish whether the observed changes in sedimentation constant could be ascribed primarily to a major modification in shape (rather than in size) of the enzyme, such as an unfolding of the protein molecule. The measurements, performed on 0.6 % amylase solutions at 3°, indicated that both the S-6 and S-4 forms of the enzyme have very close relative viscosities, namely, 1.048 and 1.041 respectively[*]. Using the relation[9]:

$$M = \frac{4690\,(S_{20,\,w})^{3/2}[\eta]^{1/2}}{(1 - \bar{V}\rho)^{3/2}}$$

and assuming identical partial specific volumes $\bar{V}$ and densities ϱ for the two forms of the enzyme, it can be calculated that a 3 fold increase in intrinsic viscosity should have occurred to account for a decrease in sedimentation constant from 6 to 4 without any change in molecular weight. This is hardly compatible with the values reported above for the relative viscosities, even though the measurements were not extrapolated to zero concentration. Therefore, it can be concluded that an actual *dissociation* of the molecule of *B. subtilis* amylase is induced by metal-binding agents.

Reversibility of the S-6 to S-4 transformation

The influence of various metal ions on the reconversion of the dissociated amylase molecule to the original S-6 form was studied. Calcium, the metal responsible for the activity and stability of α-amylases, is not involved in this transformation: a sample of amylase in the S-4 form, dialyzed for 22 h *vs.* large amounts of 0.005 M Ca^{++} ions, did not undergo any changes in sedimentation velocity or homogeneity. Furthermore, dissociation of bacterial amylase proceeds readily upon incubation with Ca-EDTA, even in the presence of a large excess of Ca^{++} ions. These results suggest that the metals required for the reassociation of the enzyme have a much stronger affinity for EDTA than has calcium, and therefore, the effect of metal ions from the first transition series[**] was investigated. Several samples of bacterial amylase in the dissociated form ($S_{20} = 4.2$) were dialyzed overnight against the usual NaCl–GP buffer to which the following metal ions were added at concentrations ranging from $2 \cdot 10^{-4}$ to $5 \cdot 10^{-3}$ M: Mn^{++}, Ni^{++}, Co^{++}, Cu^{++}, and Zn^{++}. The final concentration of enzyme was 6.0 mg/ml. In all cases, some degree of reassociation occurred, as indicated by the formation of an asymmetric leading edge on the sedimentation pattern and/or by an increase in sedimentation constant. With Mn and Ni the sedimentation constant remained below 4.5, whereas with Co and Cu, values ranging from 4.5 to 5.7 S were obtained. Of all the metals tested, Zn was by far the most effective in bringing about the reassociation of bacterial amylase. Indeed, dialysis against 0.2 to 0.5 mM Zn solution followed by a second dialysis *vs.* non-sequestering buffers in order to remove the excess of zinc resulted in the restoration of the original homogeneous S-6 sedimentation pattern*, characteristic of crystalline *B. subtilis* α-amylase. By contrast to the effects produced by Mn, Ni, Co, and Cu, the sedimentation pattern obtained under the above conditions was very reproducible**.

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[*] For comparative purposes, the viscosity increment (0.015) due to the presence of 0.01 M EDTA in the test system has been deducted from the observed relative viscosity of the S-4 amylase.

[**] In this report copper and zinc have been classified with the transition elements. This is permissible since the former two metals share many properties with the elements of the first transition series, in spite of the fact that both copper and zinc have their inner electronic orbitals filled to capacity and are therefore not true transition elements[10].

Aluminum salts, which were shown to induce the cross-linking of conarachin II[11], were without effect on the sedimentation pattern of bacterial amylase.

Role of zinc in the association of bacterial amylase

The correlation between the binding of zinc and the association of *B. subtilis* amylase has been investigated by means of radioactive ^{65}Zn-labeled enzyme, prepared as follows: A 1% solution of three times crystallized *B. subtilis* α-amylase was incubated for 1 h in 0.02 M EDTA (or Ca-EDTA) to remove the zinc originally present and then dialyzed overnight to remove the metal-binding agent. The resulting solution of dissociated S-4 amylase was incubated for 1 h with *ca.* 65–130 µg ^{65}Zn/ml, corresponding to a 10 to 20-fold molar excess of Zn over the S-6 form of the enzyme (molecular weight 97,000, see ref. [3]). The excess zinc was then removed by a second dialysis *vs.* 0.1 M NaCl + 0.02 M Na-acetate. Ultracentrifugal analysis indicated that the material obtained was in the S-6 form. Computation of the counts retained by the protein, as well as chemical metal analyses***, showed that 1.0 ± 0.1 gram-atom of zinc had been incorporated into the S-6 molecule during reassociation. Conversely, complete release of ^{65}Zn from the S-6 protein and dissociation of the latter to the S-4 form was obtained upon incubation of the ^{65}Zn-labeled amylase with Ca-EDTA and dialysis as described above.

An attempt was made to establish a parallelism between the release of zinc from the S-6 amylase and the dissociation of the enzyme. "Takamine" *B. subtilis* amylase did not lend itself to this type of analysis since this enzyme binds zinc rather strongly and does not yield readily mixtures of enzyme molecules in the associated and dissociated forms. On the other hand, "Biolase" *B. subtilis* amylase, which is apparently identical in all other respects to the former enzyme[6], was found to be more suitable for this study since it binds zinc much less firmly§. Radioactive ^{65}Zn-labeled "Biolase" amylase prepared as described above was dialyzed against a non-sequestering buffer.

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[*] Failure to eliminate the excess Zn has resulted in sedimentation constants as large as 6.7, suggesting that higher degrees of association than the one corresponding to the S-6 form of bacterial amylase can be obtained.

[**] The possibility that traces of zinc contaminating the salts of Mn, Ni, Co, and Cu were responsible for the partial associations observed should not be ruled out. Indeed, the reassociation experiments were carried out by dialysis against large volumes of solution of the various metals, and it is not known whether the enzyme can bind zinc selectively or not.

[***] The authors are indebted to Mr. J. A. RUPLEY for the zinc analyses, performed according to a method previously described[12].

[§] For instance, a 48-h dialysis of the "Takamine" amylase at pH 7.0 *vs.* a non-sequestering buffer removed none of the bound zinc, whereas the same treatment removed 80% of the zinc from the "Biolase" preparation. It is not known whether this results from a different structure of the zinc-binding site of the *native* amylase molecule or from modifications that have occurred during the preparation of the starting material "Takamine" or "Biolase".

As the zinc content fell below 1 gram-atom/mole of S-6 amylase, heterogeneous sedimentation patterns were obtained (Fig. 2). The proportion of S-6 and S-4 molecules was determined, after resolution of enlarged projections of these diagrams, according to OGSTON[18]. The parallelism between the loss of zinc and the dissociation of the enzyme molecule is illustrated in Fig. 3.

Fig. 2. Sedimentation pattern of zinc-depleted "Biolase" amylase. Direction of sedimentation: from right to left. (a) 55% associated molecules; 45% dissociated molecules. The exposure was taken with a bar angle of 40°, 120 min after the rotor had reached full speed. (b) 35% associated molecules; 65% dissociated molecules. The exposure was taken with a bar angle of 35°, 150 min after the rotor had reached full speed.

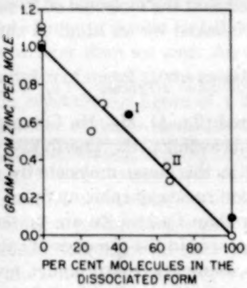

Fig. 3. Correlation between the release of zinc from *B. subtilis* α-amylase and the dissociation of the enzyme molecule. A 1% solution of ^{65}Zn-labeled "Biolase" amylase containing 0.67 μg Zn (790 counts/min) per mg protein, corresponding to a 1:1 molar ratio of Zn to dimer amylase (mol. wt. 97,000) is dialyzed against 0.1 *M* NaCl–0.02 *M* Na-acetate at 5°, pH 7.0. At various times, aliquots are removed, analyzed for zinc, and examined in the ultracentrifuge. ○, ^{65}zinc content computed from radioactivity measurements. ●, zinc content determined by chemical analysis. The sedimentation diagrams corresponding to points I and II are illustrated in Figs. 2a and 2b respectively.

In view of the weak binding of zinc by the "Biolase" amylase, labeling of the protein can also be achieved by direct addition of ^{65}Zn to the enzyme in the S-6 form. For the crystallization of the ^{65}Zn-labeled amylases ("Takamine" as well as "Biolase"), enzyme solutions of at least 15 mg/ml are dialyzed for approximately 6 h against 0.02 *M* NaCl and 0.004 *M* Na-acetate, pH 7.2, to remove the excess zinc. Calcium acetate (0.01 *M*) is then added and the pH adjusted to 6.8. As reported previously[3], the dissociated form of the enzyme does not crystallize under these conditions.

Dissociation of bacterial amylase in the presence of urea

In an attempt to obtain bacterial amylase sub-units of still lower sedimentation constant, the enzyme was dialyzed *vs.* 0.01 *M* EDTA in 6.6 *M* urea. Ultracentrifugal analysis, after removal of EDTA and urea by dialysis, indicated that the S-6 to S-4 dissociation had proceeded as usual, without further *irreversible* transformations due to the denaturing agent. Since this enzyme contains no cysteine or cystine residues[14, 15], no attempts were made to split the molecule with sulfhydryl reagents such as *p*-chloromercuribenzoate or sodium borohydride. The S-4 molecule can therefore be regarded as being the true monomer of *B. subtilis* amylase, a conclusion supported by the amino acid analysis of this enzyme[15].

Ultracentrifugal analysis of other α-amylases

Pure crystalline α-amylases from human saliva, hog pancreas, and *A. oryzae* are found, under normal conditions, only in the S-4 form[16–18]. Their sedimentation pattern remains unaffected by addition of zinc or EDTA.

DISCUSSION

All α-amylases so far investigated, including bacterial amylase, are believed to be *calcium metalloenzymes*[5]. The apparent intrinsic association constant of calcium for *B. subtilis* amylase is of the order of magnitude of 10^{12} to 10^{15}, and this metal will not be released under the experimental conditions used in this study. Zinc, on the other hand, which has been found in significant amounts in *B. subtilis* amylase only[19], is bound much less firmly. Sedimentation and diffusion constant measurements on S-6 and S-4 *B. subtilis* α-amylase* indicate that the size of the S-6 molecule is exactly twice that of the S-4 molecule. Since dimerization requires the uptake of one atom of zinc, which is released upon dissociation, it is proposed that the molecule of *B. subtilis* α-amylase is constituted of two monomer units crosslinked by an atom of zinc according to the following scheme:

$$[\text{Protein} - \text{Ca}_x] - \text{Zn} - [\text{Protein} - \text{Ca}_x] \underset{\text{Zn}^{++}}{\overset{\text{Metal binding agents}}{\rightleftharpoons}} 2[\text{Protein} - \text{Ca}_x] + \text{Zn}$$

In spite of the fact that none of the cations tested (Ca, Al, Mn, Ni, Co, and Cu) was as effective as zinc in forming intermolecular crosslinks, the possibility that, under special circumstances, zinc could be replaced in the dimer molecule by some other cation has not been ruled out. However, emission spectrographic analyses have shown that no significant quantities of metals other than Ca and Zn are present in pure bacterial amylase[19]. Since dimerization does not occur in the presence of calcium alone, it can be concluded that purified *B. subtilis* α-amylase exists under normal conditions in the form of a zinc–protein complex. Dimerization under the influence of zinc and formation of a zinc–protein complex appears to be peculiar to *B. subtilis* α-amylase. However, the remarkable effect of divalent metals in promoting the crystallization of other α-amylases should be kept in mind. For instance, the striking decrease in the apparent solubility of human saliva amylase caused by Ni^{++} ions could be taken to indicate that a metal-induced aggregation is necessary for crystallization[3, 19].

The biological role of zinc in bacterial amylase is not clear. This metal is apparently not required for the protection of the enzyme against proteolytic degradation[2], and there is no evidence that zinc is involved in the activity of the enzyme. Further studies are underway to determine whether dissociation of the zinc-containing dimer is required for the formation of the enzyme–substrate complex.

Preparations of *B. subtilis* α-amylase exhibit their highest degree of homogeneity in the ultracentrifuge when maintained in the monomer state by an appropriate sequestering agent. Special precautions against metal contamination must be taken to maintain the enzyme in the dissociated form in the absence of metal-binding agents, and such preparations should be stored in polyethylene tubes rather than in glass containers. A truly homogeneous dimer amylase is more difficult to obtain on account of (a) further polymerization that may take place in the presence of an excess of zinc, (b) the tendency of zinc ions to be converted to the hydroxide at the pH of optimum stability of the enzyme (see ref. [10], p. 502), and (c) the lability of the zinc complex, particularly in the alkaline range of pH where most buffers possess metal-binding properties. Careful examination of the S-6 sedimentation pattern described in Table I indicates that even at pH 7.0 a very slightly skewed trailing edge is present when GP is used as a buffer, in contrast to the perfectly symmetrical edges obtained in acetate buffer or in NaCl.

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* The details of this study will be published.

Striking similarities exist between the effect of transition elements on *B. subtilis* α-amylase and those described by CUNNINGHAM *et al.*[20] in a study of the binding of zinc and cobalt by insulin. As in the case of "Takamine" amylase (a) small amounts of zinc cause a rapid rise in the sedimentation constant of amorphous insulin, corresponding to an association of the protein sub-units, (b) the zinc taken up by the hormone during the first stage of its polymerization (*i.e.*, up to $S_{20} = 3.5$ which corresponds to the hexamer form of insulin of molecular weight 36,000, see ref. 21) is not released upon dialysis *vs.* non-sequestering buffers, (c) increasing amounts of bound zinc lead to higher states of aggregation, which dissociate readily upon dialysis, and (d) the affinity of insulin for other transition elements (*e.g.*, Co^{++} and Fe^{++}) seems to be much weaker than for zinc. Among the various values reported in the literature for the number of metal atoms present in the unit cell (weight *ca.* 35,000) of rhombohedral insulin crystals, the figure of 3 atoms per cell is of particular interest since it is also the number of dimer elements within the cell[22]. In any event, zinc greatly increases the crystallizability of both insulin and bacterial amylase, and SCHLICHTKRULL has claimed[22] that 2 atoms per unit cell represent the minimum content of zinç ions sufficient for crystallization of insulin.

Since a preliminary report was made on the role of zinc in bacterial amylase[1], FRIEDEN[23] has reported that glutamic dehydrogenase from beef liver is constituted of 4 monomer units held together by zinc. Dissociation of this zinc-protein complex could be obtained not only by means of chelating agents but also by addition of the co-substrate for the enzyme, namely diphosphopyridine nucleotide. How general will be the structural function of zinc in the crosslinking of protein subunits must await further investigation.

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STUDIES ON BACTERIAL CHROMATOPHORES

I. REVERSIBLE DISTURBANCE OF TRANSFER OF ELECTRONIC EXCITATION ENERGY BETWEEN BACTERIOCHLOROPHYLL-TYPES IN *CHROMATIUM*

C. BRIL

Biophysical Research Group, Physical Institute of the State University, Utrecht (The Netherlands)

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SUMMARY

Desoxycholate affects the transfer of excitation energy between bacteriochlorophyll-types in *Chromatium* chromatophores. It is suggested, that the detergent fragments the chromatophore into subunits and subsequently disturbs the spatial arrangement of these bacteriochlorophyll-types.

INTRODUCTION

Soon after the isolation of pigmented submicroscopic particles from purple bacteria[1], it became probable that in these structures the complete photosynthetic apparatus of these organisms is localized. Photochemical activities and light-induced phosphorylations could be demonstrated and recently anaerobic photophosphorylation coupled to light-induced oxidation-reduction reactions was reported[2]. Functionally, therefore, bacterial chromatophores seem to be comparable with chloroplasts of higher plants.

About the ultrastructure of the chromatophores very little is known. As they are mainly composed of phospholipoprotein complexes, which have resisted attempts of separation and isolation by various methods[3], a highly organized structure is indicated. The presence of lamellae was indicated by electron microscopy[4,5]. The *in vivo* state of bacteriochlorophyll, and of photosynthetic pigments in general, still is an unsolved problem. Several lines of indirect evidence suggest an association of these pigments with some other substance, probably a lipoprotein. For a recent review bearing on this problem, see ref. [6].

For a study of the state of chlorophyll in its functional environment, the purple bacteria are particularly interesting. Their bacteriochlorophyll-carrier complexes show a large absorption shift towards the infrared (from 780 to 890 mμ), when compared with bacteriochlorophyll in organic solvents. Moreover, their chlorophyll, which has been shown to be identical for all species, exhibits *in vivo* absorption properties which are strikingly different for various strains, even within a single strain. As these spectral properties have been described many times before (*cf.* [7]), it may suffice here to mention the intriguing phenomenon of some species showing two or more additional absorption peaks in the 800–850 mμ region. This multiplicity of peaks is supposed to be caused by different modes of associations of bacteriochlorophyll with one or more specific carrier molecules. To avoid any suggestion as to the nature of these complexes, they have been named bacteriochlorophyll-types[8].

To enable a more direct study of these types by disruption of their structural organization and, if possible, to isolate them in a physiologically active state, it is necessary to choose mild solubilizing procedures, as the pigment is easily detached from its natural complex. For this purpose detergents, known to have a solubilizing effect on systems rich in lipides, have been used. Preliminary experiments on the action of a non-ionic detergent on chromatophores of the Athiorhodacea *Rhodopseudomonas spheroides* showed the usefulness of such a procedure[9]; the results suggested that one of the bacteriochlorophyll-types could be split off. Unfortunately, however, chromatophores of this species proved to be rather unstable in the presence of the detergent used. Gradual spectral changes are taking place upon standing, even in the dark.

It was found that chromatophores of the Thiorhodacea *Chromatium*, another species with three absorption maxima in the near infrared, behave quite differently. When suspended in desoxycholate, which is without any perceptible effect upon chromatophores of *Rhodopseudomonas*, their pigment systems are stable when kept in the dark and with certain precautions no absorption changes occur upon illumination[10].

Chromatium shows three absorption maxima at approximately 800, 850 and 890 mμ. The bacteriochlorophyll-types responsible for these absorptions will be termed B_{800}, B_{850} and B_{890}, respectively. The fluorescence spectrum of the organism, however, only shows a major peak at about 910 mμ and B_{890} seems to be the only fluorescent pigment type. The fluorescence spectrum is independent of the wavelength of the exciting light. Light quanta absorbed by B_{800} give rise to fluorescence of molecules of B_{890} with about the same efficiency as do quanta absorbed by B_{890} itself[8]. When the absorption maximum at 890 mμ, recognizable as a shoulder, is made to disappear by heating, a new fluorescence peak at 880 mμ occurs, indicating the capacity of B_{850}

to emit fluorescence. This fluorescence is absent in the intact organism. These findings lend strong support to the concept of transfer of excitation energy between photosynthetic pigments and in *Chromatium* transfer of excitation energy of high efficiency may take place from B_{800} and probably from B_{850} to B_{890}, which seems to be the photosynthetically active pigment system in bacterial photosynthesis[8]. Since the efficiency of energy transfer approaches 100 %, the mechanism of this transfer is presumed to be that of inductive resonance. As the efficiency of this energy transfer is inversely proportional to the sixth power of the distance between the types of molecules[11], changes in the spatial arrangement of the various types in the chromatophore might result in considerable changes in the efficiency of this transfer. Consequently, upon illumination with light which is absorbed by all three types simultaneously, changes in the fluorescence spectrum of structurally disorganized chromatophores may be expected. In the first place the intensity of the fluorescence emitted by B_{890} would be expected to decrease, as it is no longer supplied with energy absorbed by the other types. The B_{850} material on the other hand, no longer able to transfer its excitation energy to B_{890}, will emit part of this energy as fluorescence, resulting in an increase in fluorescence intensity at 880 mμ. If B_{800} is also separated from the other types, it would likewise become able to emit measurable fluorescence and increases of fluorescence intensities around 830 mμ may be envisaged. Under suitable conditions such changes may indeed be induced in the fluorescence pattern of chromatophores by desoxycholate treatment.

MATERIALS AND METHODS

Chemicals and enzyme preparations

Chymotrypsin type II and tris(hydroxymethyl)aminomethane were obtained from the Sigma Chemical Company, crystalline trypsin from Worthington Biochemical Corporation, snake venom (*Crotalus atrox*) from Ross Allen's Reptile Institute, Silver Springs, sodium desoxycholate from the British Drug Houses and L-malate from Nutritional Biochemicals Corporation.

Throughout the experiments 0.05 *M* Tris buffer pH 8.5 was used.

Isolation of chromatophores

Chromatium strain D was grown in a malate-thiosulphate medium. The spectrum of the organism may vary considerably upon subculturing. The relative height of the 850 mμ peak is especially liable to show marked changes. Reduction of absorption is accompanied by a shift of the absorption maximum towards shorter wavelengths. These changes are not predictable. After periods of relative stability, organisms with either a reduced or increased absorption at about 850 mμ may be harvested. In Fig. 1 an example of a spectrum with a high 850 mμ absorption is given. This phenomenon has been described before[12] and its cause is still obscure.

After harvesting and washing with buffer the bacterial cells were disrupted by grinding with carborundum. This method was prefered to disruption by sonic vibration to avoid possible damage to the chromatophore[13]. Whole cells and cell debris were removed by 3 to 4 successive centrifugations of 20 min duration at 10,000 × *g* and the supernatant once more centrifuged at 25,000 × *g* for 15 min. Finally, the chromatophores were spun down at 144,000 × *g* during 1 h and the sediment was homogenized in a Potter-Elvehjem homogenizer. Concentrated suspensions of chromatophores thus obtained can be stored in a refrigerator in the dark for several weeks without spectral changes occurring.

All preparations used in the experiments were adjusted to an optical density of 2.5–3 (1 cm path length) at 800 mμ, corresponding with a bacteriochlorophyll concentration of 50–60 μg/ml.

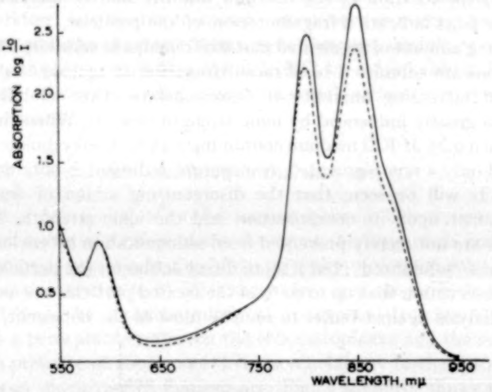

Fig. 1. Spectra of chromatophores in buffer (solid line) and in 0.25 % desoxycholate–0.25 *M* KCl (dotted line).

Apparatus

The apparatus for the measurement of fluorescence was the same as described earlier[14], with only slight modifications to improve sensitivity and stability. Fluorescence was excited by irradiation with light of 592 mμ obtained by passing light from a 100 W incandescent lamp through a GAB interference filter. This wavelength was chosen, as a minor absorption band of the three bacteriochlorophyll-types coincides at 590 mμ. Thus, this band is well separated from the infrared peaks. The absorption of a type at this wavelength is proportional to its absorption at the infrared peak[8]. After elimination of scattered light by means of a Kodak Wratten filter 87, the fluorescent light was focussed on an infrared sensitive Maurer-photomultiplier cooled with liquid air. The signals were fed into a Honeywell-Brown recorder. The cuvette containing the extracts had a width of 0.8 mm. By replacing different filters in front of the photomultiplier, the fluorescence at different wavelengths was determined. For measuring B_{890} fluorescence beyond 900 mμ a bacterial-filter (BF) was used[13].

As these have been found to be negligible in our experiments with partially purified chromatophores, scattering corrections have not been applied. To ascertain these corrections heated and subsequently illuminated extracts of chromatophores have been used. Upon heating, absorption in the 800–890 mμ region disappears and a new peak at about 780 mμ arises. The material responsible for this absorption emits a fluorescence, which is quenched upon illumination. Extracts thus treated did not give rise to any significant signals when irradiated with 592 mμ light.

RESULTS

Absorption and sedimentation characteristics of desoxycholate-treated particles

When desoxycholate is added to a buffered suspension of chromatophores, an instant clarification occurs. This increase of light transmission is demonstrated in the absorption spectrum of desoxycholate-treated chromatophores (Fig. 1). Slight shifts of a few mμ of the location of the maxima towards shorter wavelengths are also observed. These facts indicate a fragmentation of the particles.

An increasing amount of pigmented material remains in solution, when chromatophore suspensions are submitted to ultracentrifugation at 144,000 × *g* for 60 min in the presence of increasing amounts of desoxycholate. This solubilizing effect of desoxycholate is greatly influenced by ionic strength (Fig. 2). When chromatophores are suspended in a 0.25 *M* KCl medium containing 0.25 % desoxycholate solubilization is complete and only a non-pigmented, transparant sediment is obtained upon ultracentrifugation. It will be seen, that the disorganizing action of desoxycholate is similarly dependent upon its concentration and the ionic strength. Therefore, the chromatophores are not merely prevented from sedimentation by enclosing detergent micelles, and thus "solubilized", but a more direct action on the particles seems to be involved. The observation that up to 90 % of the treated particles are not sedimented after rigorous dialysis against buffer to remove most of the detergent, supports this view.

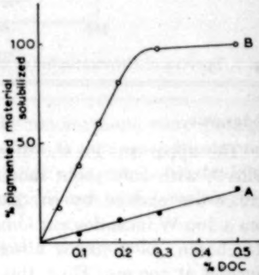

Fig. 2. Influence of ionic strength on sedimentation of chromatophore material at 144,000 × *g* at increasing desoxycholate concentration. A. without KCl; B. in the presence of 0.25 *M* KCl.

The effect of desoxycholate on transfer of excitation energy between bacteriochlorophyll-types

The changes in the fluorescence pattern of chromatophore suspensions in the 800–910 mμ region due to desoxycholate action are shown in Table I, A and B, which represents the results of an arbitrary experiment. A relatively small decrease of B_{890} fluorescence is induced by the detergent, while a significant increase of B_{850} fluorescence is shown. This increase was observed in all preparations tested. The slight decrease of B_{890} fluorescence, however, did occur in most cases, but not in all. The changes of this fluorescence were small in any case, which may be explained as being due to an overlap of the B_{850} and B_{890} fluorescence spectra and of the transmission of the used filters. Consequently, a fraction of the fluorescence emitted by B_{850} is also measured. This explanation is consistent with the observation, that there is a correlation between sign and magnitude of the changes of B_{890} fluorescence intensity and the relative height of the 850 mμ absorption band of the corresponding extracts. In extracts with a high

850 mμ absorption sometimes even a slight increase of this fluorescence intensity was measured. Some reabsorption of fluorescence emitted by B_{850} also occurs, but this effect is of minor importance.

Though no quantitative significance should be attached to the values given in Table I, the value for the fluorescence intensity of detergent-treated particles at 807 mμ is notably low. This could be due to either the emission by B_{800} of a fluorescence of low quantum yield or the fact that B_{800} is not spatially separated from the other types and, therefore, is still able to transfer its excitation energy, probably to B_{850}. Of these two possibilities the latter seems to be the most likely[10].

Extracts in the presence of desoxycholate-KCl are remarkably stable as they may be stored for several days in a refrigerator in the dark without any changes in absorption and fluorescence.

Influence of pH, desoxycholate concentration and ionic strength

The action of desoxycholate on the chromatophore is independent of pH between 7.5 and 10. For a maximal effect of the detergent the ratio of its concentration and KCl concentration is critical. In Fig. 3 the influence of desoxycholate concentration at a fixed KCl concentration (0.25 *M*) is shown for chromatophore suspensions diluted as described before. At a concentration of approximately 0.075 %, the detergent starts to exert a perceptable action on the chromatophores and the maximal effect is reached in the presence of about 0.25 % desoxycholate. A further increase in concentration results in quenching of fluorescence. Fig. 4 similarly shows the influence of ionic strength at a fixed detergent concentration (0.25 %). No quenching of fluorescence occurs at high KCl concentration.

In these experiments cysteine has been added to the extracts as a precautionary measure to prevent photooxidation of bacteriochlorophyll as well as light-induced quenching of fluorescence[10].

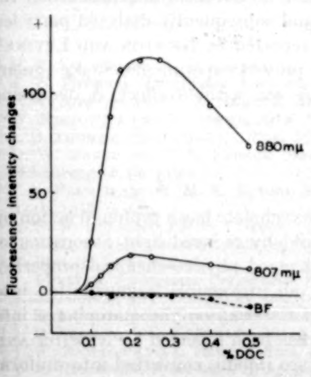

Fig. 3. Influence of desoxycholate (DOC) concentration at a fixed KCl concentration (0.25 *M*) on the fluorescence intensities of chromatophore suspensions at different wavelengths (mμ).

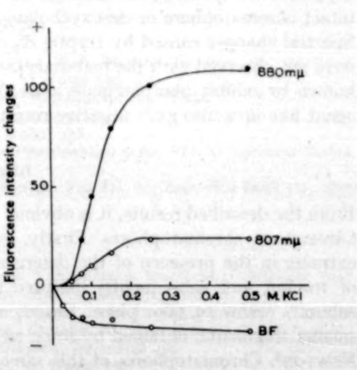

Fig. 4. Influence of ionic strength at a fixed desoxycholate concentration (0.25 %) on the fluorescence intensities of chromatophore suspensions at different wavelengths (mμ).

Reversibility of the disorganizing action of desoxycholate on chromatophores

The effect of desoxycholate with respect to the disturbance of transfer of excitation energy between bacteriochlorophyll-types is fully reversible. Dialysis of extracts of detergent-treated particles results in restoration of the original fluorescence pattern of the chromatophores. Subsequent readdition of desoxycholate and KCl reproduces the action of the detergent (Table I, C and D). Optimal results were obtained, when the extracts were dialyzed in Visking cellulose tubing for 12 h against 1 l change of buffer. Dialysis against buffer of high ionic strength (0.25 *M* KCl) causes the chromatophores to flocculate upon standing, indicating that also some irreversible changes of the chromatophores have been induced by detergent action. This phenomenon is in accordance with ultracentrifugation studies of dialyzed extracts described earlier.

TABLE I

RELATIVE FLUORESCENCE INTENSITIES OF CHROMATOPHORE SUSPENSIONS UNDER VARIOUS CONDITIONS AT DIFFERENT WAVELENGTHS (mμ)

	BF + y	*880*	*807*
A. In buffer	63	29	2
B. In 0.25% desoxycholate–0.25 *M* KCl, before dialysis	48	117	19
C. Same preparation as B., after dialysis	59	30	2
D. Same preparation as C., desoxycholate and KCl readded	42	109	18

Fluorescence intensities are given in terms of recorder deflections in mm.

Failure to affect the spatial arrangement of bacteriochlorophyll-types by other chemical and enzymic methods

So far we have failed to obtain similar results as described above by other methods. Trypsin and chymotrypsin, alone or in combination, do not seem to attack either the intact chromatophore or desoxycholate-treated and subsequently dialyzed particles. Spectral changes caused by tryptic digestion as reported by NEWTON AND LEVINE[15] were not observed with the materials used in the present experiments. Snake venom, known to exhibit phospholipase activity, likewise was without effect. A dispersing agent like urea also gave negative results.

DISCUSSION

From the described results, it is obvious that desoxycholate has a profound action on *Chromatium* chromatophores. Firstly, as evidenced by reduced light absorption of extracts in the presence of the detergent and changed physico-chemical properties of treated and subsequently dialyzed particles, an irreversible fragmentation into subunits seems to take place. Fragmentation of *Chromatium* chromatophores into smaller fragments, obtained by sonic treatment, has been reported by NEWTON AND NEWTON[3]. Chromatophores of this same species are rapidly converted into uniform particles, which sediment much less rapidly upon cyanide treatment[16]. It may be mentioned here, that 1 *M* KCN does not affect the spatial arrangement of the bacteriochlorophyll-types. Beautiful electron micrographs of intact chromatophores and of subunits of *Rhodospirillum rubrum* were published by FRENKEL[2], but the method by which these subunits were obtained was not specified. The complete photosynthetically active system seems to be present in these subunits. This assumption is suggested by the following observations: (1) their spectrum shows the presence of the bacteriochlorophyll-types, (2) when they are obtained by sonic desintegration, transfer of excitation energy is not affected[13] and (3) they are as active photochemically as the larger chromatophore[2].

These data are consistent with the view that chromatophores are made up of assemblies of identical subunits, which may be orderly arranged, *e.g.* in lamellae. Desoxycholate treatment, as does sonic rupture, seems to achieve fragmentation along fragile lines of attachment.

Secondly, desoxycholate has an additional reversible action on the released subunits, demonstrated in changes in transfer of excitation energy, which, among others, requires certain spatial arrangements. Probably a penetration of detergent molecules into the fragments occurs, affecting the spatial relationships of the bacteriochlorophyll-types and thus separating them over a distance too great for energy transfer. This does not seem to involve gross changes in the chemical and physico-chemical state of these complexes, as these would notably affect absorption.

The experimental results may be tentatively explained by assuming that B_{890} is reversibly distantiated from B_{800} and B_{850} while these latter two types still may have retained their natural complexity. As additional manifestations of the action of desoxycholate on chromatophores will be described in a subsequent paper, a further discussion will be postponed until then.

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MECHANISM OF INHIBITION OF D-AMINO ACID OXIDASE

III. KINETIC ANALYSIS OF THE BEHAVIOUR OF CHLORAMPHENICOL, STREPTOMYCIN AND PENICILLIN IN THEIR COMPETITION WITH FLAVIN-ADENINE DINUCLEOTIDE

KUNIO YAGI AND TAKAYUKI OZAWA
Department of Biochemistry, School of Medicine, Nagoya University, Nagoya (Japan)

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SUMMARY

1. A kinetic method using FMS and AMS as indicators was devised for the analysis of the behaviour of an inhibitor in its competition with the co-enzyme FAD.

2. Chloramphenicol, streptomycin and penicillin were found to inhibit D-amino acid oxidase by combining with the apo-protein in competition with the co-enzyme. The dissociation constants of their complexes with the oxidase protein in competition with FAD were calculated to be $2.1 \cdot 10^{-3}$ *M*, $1.8 \cdot 10^{-2}$ *M* and $1.4 \cdot 10^{-2}$ *M*, respectively.

3. All three antibiotics competed with the AMP part of the FAD.

4. The cause of ariboflavinosis induced by the administration of an antibiotic is discussed.

INTRODUCTION

In previous papers[1], it has been demonstrated that chlortetracycline inhibits D-amino acid oxidase activity by forming a complex with FAD in competition with the oxidase protein, and the question was raised whether the formation of a complex by chlortetracycline and flavin might not partly explain the mechanism of the manifestation of ariboflavinosis or ariboflavinosis-like disease caused by the administration of this antibiotic.

In the field of clinical medicine, an ariboflavinosis-like disease has also been reported as a side effect of the administration of chloramphenicol[2-4], streptomycin[5,6], or penicillin[7-9]. It was thought possible that ariboflavinosis was brought about by the inhibitory action of these antibiotics on the flavin enzyme. Using D-amino acid oxidase as an example of a flavin enzyme, the inhibitory action of these antibiotics in competition with FAD was demonstrated and investigated by a kinetic method, which indicated that they act on the oxidase protein.

Abbreviations: FAD, flavin-adenine dinucleotide; FMN, flavin monophosphate; AMP adenosine monophosphate; FMS, riboflavin 5′-monosulfate; AMS, adenosine 5′-monosulfate.

MATERIALS

D-Amino acid oxidase protein, free from FAD, was prepared by the method of NEGELEIN AND BRÖMEL[10]. The enzyme solution containing 15 μg of the oxidase protein per ml of pyrophosphate buffer (0.1 M, pH 8.3) was used for the experiments reported in this paper. 1 ml of it could catalyse 132 μl of oxygen uptake/30 min at 38° in the presence of a large excess of both DL-alanine and FAD. When no FAD was present, no oxygen uptake was observed.

FAD was prepared by the method of YAGI *et al.*[11] and was over 92 % pure; it contained no other flavins, nucleic acids or metals.

FMS and AMS were synthesized by the method of TAKAHASHI, YAGI AND EGAMI[12], and YAMASHINA AND EGAMI[13], respectively.

Chloramphenicol, dihydrostreptomycin sulphate and benzylpenicillin sodium were supplied by Park Davis Ltd., Pfizer Ltd., and Takeda Pharm. Ind. Ltd., respectively.

METHODS

Complex formation of an antibiotic with FAD

Spectrophotometry and fluorimetry were used for the detection of complex formation. The test solution contained $1.5 \cdot 10^{-6}$ M of FAD and a gradually increasing concentration of antibiotic. A possible shift of absorption spectrum and quenching of fluorescence of FAD were examined at 38° and pH 8.3.

Measurement of oxidase activity

The oxidase activity was measured by a conventional Warburg manometer at 38° and pH 8.3. The gas phase was air, and the centre well contained 0.2 ml 20 % NaOH.

In investigating the competitive inhibition of an antibiotic with FAD, the reaction mixture contained 15 μg of the oxidase protein, excess of DL-alanine, a rate-limiting concentration of FAD (final concentration, $1.7 \cdot 10^{-7}$ M) and a gradually increasing concentration of an antibiotic in pyrophosphate buffer (0.1 M, pH 8.3).

The competitive inhibition of an antibiotic with the substrate was examined using a reaction mixture containing the oxidase protein, excess of FAD, a rate-limiting concentration of D-alanine (final concentration, $2.1 \cdot 10^{-3}$ M) and a gradually increasing concentration of an antibiotic. Only chloramphenicol was found to inhibit the oxidase activity in competition with the substrate, and the dissociation constant of the complex of chloramphenicol and the oxidase protein was calculated to be $1.0 \cdot 10^{-3}$ M using Methods I and II described below.

Kinetic study for the competition of an inhibitor with FAD

The dissociation constants of D-alanine (K_s) and FAD (K_f) with the oxidase protein were calculated to be $3.3 \cdot 10^{-3}$ M, and $1.1 \cdot 10^{-7}$ M, respectively, using the Michaelis-Menten formula.

Assuming that an antibiotic forms a complex with the protein in competition with FAD, and that its dissociation constant with the protein (K_{ip}) is sufficiently larger than K_f, the enzyme activity can be shown as

$$v = \frac{V f}{K_f(1 + i/K_{ip}) + f} \tag{1}$$

where v is the reaction velocity in the presence of f concentration of FAD, V is the maximum velocity reached in the presence of a large excess of FAD, and i is the concentration of an antibiotic.

Using the Lineweaver-Burk form of eqn. (1), K_{ip} can be evaluated by plotting $1/v$ against $1/f$ (Method I).

From the Michaelis–Menten formula and eqn. (1), the following equation can be derived,

$$\frac{v_0}{v} = 1 + \left\{1 - \frac{v_0}{V}\right\}\frac{i}{K_{ip}} \tag{2}$$

where v_0 and v are the reaction velocities in the absence and presence of an antibiotic, respectively. If the values of v_0/v plotted against i give a straight line with the intercept 1, the inhibitory action can be considered to be of single mechanism as previously reported[14]. From the slope of the straight line thus obtained, K_{ip} can be calculated (Method II).

For the investigation of the competition of an inhibitor with FAD, i.e. whether an inhibitor becomes bound to the protein in competition with the AMP or the FMN part of FAD, a kinetic method using FMS and AMS as specific indicators was devised. As reported previously[15], it was observed that FMS and AMS inhibit the oxidase competitively with the FMN and AMP part of FAD, respectively. The dissociation constants of FMS and AMS with the oxidase protein were found to be $3.2 \cdot 10^{-5}$ M and $3.1 \cdot 10^{-3}$ M, respectively.

Assuming that an indicator is present together with an antibiotic which inhibits D-amino acid oxidase in competition with FAD, that these two inhibitors and FAD compete with each other for the binding site of the protein with the FMN or AMP portion of FAD, and that the antibiotic does not combine with FAD, the following treatment will be possible (Case I).

Putting

e = total concentration of the oxidase protein,
i_1 = total concentration of inhibitor 1,
i_2 = total concentration of inhibitor 2,
x = concentration of the protein–FAD complex,
m = concentration of the protein–inhibitor 1 complex,
n = concentration of the protein–inhibitor 2 complex,
K_1 = dissociation constant of the protein–inhibitor 1 complex,
K_2 = dissociation constant of the protein–inhibitor 2 complex,

and with other subscripts as already indicated, the over-all reaction may be written as,

$$\underset{(e-x-m-n)}{E} + \underset{(f)}{F} \overset{K_f}{\rightleftharpoons} \underset{(x)}{EF}$$

$$\underset{(e-x-m-n)}{E} + \underset{(i_1)}{I_1} \overset{K_1}{\rightleftharpoons} \underset{(m)}{EI_1}$$

$$\underset{(e-x-m-n)}{E} + \underset{(i_2)}{I_2} \overset{K_2}{\rightleftharpoons} \underset{(n)}{EI_2}$$

In this case,

$$x = \frac{e f}{K_f(1 + i_1/K_1 + i_2/K_2) + f} \tag{3}$$

On the other hand, when two inhibitors compete with FAD, but not with each other, for example, one inhibitor competes with the FMN part of FAD and the other competes with the AMP part of FAD, and the antibiotic does not form a complex with FAD (Case II),

$$\begin{array}{ccccc}
\underset{(e-x-m-n-p)}{E} & + & \underset{(f)}{F} & \overset{K_f}{\rightleftharpoons} & \underset{(x)}{EF} \\
\underset{(e-x-m-n-p)}{E} & + & \underset{(i_1)}{I_1} & \overset{K_1}{\rightleftharpoons} & \underset{(m)}{EI_1} \\
\underset{(e-x-m-n-p)}{E} & + & \underset{(i_2)}{I_2} & \overset{K_2}{\rightleftharpoons} & \underset{(n)}{EI_2} \\
\underset{(m)}{EI_1} & + & \underset{(i_2)}{I_2} & \overset{K_3}{\rightleftharpoons} & \underset{(p)}{EI_1I_2} \\
\underset{(n)}{EI_2} & + & \underset{(i_1)}{I_1} & \overset{K_4}{\rightleftharpoons} & \underset{(p)}{EI_1I_2}
\end{array}$$

where

p = concentration of the protein–inhibitor 1, 2 complex
K_3 = dissociation constant of the complex of protein–inhibitor 1 and inhibitor 2
K_4 = dissociation constant of the complex of protein–inhibitor 2 and inhibitor 1

In this case,

$$x = \frac{e\,f}{K_f(1 + i_1/K_1 + i_2/K_2 + i_1i_2/K_1K_3) + f}$$

or

$$x = \frac{e\,f}{K_f(1 + i_1/K_1 + i_2/K_2 + i_1i_2/K_2K_4) + f} \tag{4}$$

From the Michaelis–Menten equation and eqns. (3) or (4), respectively the following equations can be derived

$$\frac{v_0}{v} = 1 + \left\{1 - \frac{v_0}{V}\right\}\left\{\frac{i_1}{K_1} + \frac{i_2}{K_2}\right\} \tag{5}$$

$$\frac{v_0}{v} = 1 + \left\{1 - \frac{v_0}{V}\right\}\left\{\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1i_2}{K_1K_3}\right\}$$

or

$$\frac{v_0}{v} = 1 + \left\{1 - \frac{v_0}{V}\right\}\left\{\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1i_2}{K_2K_4}\right\} \tag{6}$$

When the values of v_0/v are plotted against i, eqn. (5) gives a straight line, while eqn. (6) gives a second-order curve; i.e. a straight line is obtained in Case I, and a curve in Case II. Accordingly, if plots of v_0/v against i give a straight line in the presence of an antibiotic and FMS, and a second-order curve in the presence of an antibiotic and AMS, it may be concluded that an antibiotic competes with the FMN part of FAD. On the other hand, if plots of v_0/v give a second-order curve in the presence of an antibiotic and FMS, and a straight line in the presence of an antibiotic and AMS, it may be concluded that the antibiotic competes with the AMP part of FAD.

In this way, using FMS and AMS as indicators, the behaviour of an antibiotic in its binding with the protein may be analysed from the form of the curve.

RESULTS

Complex formation of antibiotics with FAD

None of the antibiotics chloramphenicol, streptomycin or penicillin cause a shift of the absorption spectrum of FAD. Streptomycin and penicillin do not quench the fluorescence of FAD. On the other hand, $6 \cdot 10^{-5}$ *M* chloramphenicol dims the fluorescence of FAD to about one half of its original intensity. However, from the fact that chloramphenicol does not shift the absorption spectrum of FAD and in view of the results on the inhibition of the oxidase by chloramphenicol as described below, complex formation between chloramphenicol and FAD may be excluded.

Inhibitory action of chloramphenicol

As shown in Fig. 1, measured values of v_0/v plotted against the concentration of chloramphenicol gave a straight line, which indicates that there could be a single inhibitory factor with respect to the FAD. By plotting $1/v$ against $1/f$, a straight line was also obtained. These results showed that this antibiotic competes with FAD for the oxidase protein. Using Methods I and II, K_{ip} was calculated to be $2.1 \cdot 10^{-3}$ *M*.

Fig. 1 shows that FMS together with chloramphenicol gave a curve, while AMS together with the antibiotic gave a straight line. This indicates that chloramphenicol competes with the AMP part of FAD.

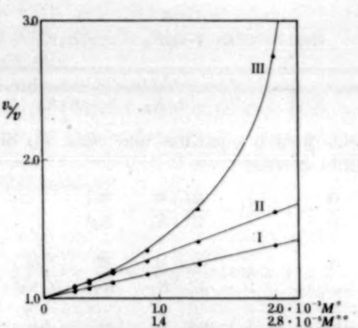

Fig. 1. Analysis of the inhibitory action of chloramphenicol on D-amino acid oxidase, using kinetic indicators. v_0 and v represent the reaction velocities in the absence and presence of inhibitors. The reaction mixture contained 15 μg of the oxidase protein, 0.15 *M* of DL-alanine and $1.7 \cdot 10^{-7}$ *M* of FAD. * chloramphenicol concn., ** FMS concn., *** AMS concn. Curve I: in the presence of chloramphenicol. Curve II: in the presence of chloramphenicol and AMS. Curve III: in the presence of chloramphenicol and FMS. *V* was 132 μl oxygen uptake/30 min and v_0 was 80 μl oxygen uptake/30 min.

Inhibitory action of streptomycin

Since streptomycin does not form a complex with FAD and straight lines were obtained in both Methods I and II (see line I, Fig. 2), it was considered that this antibiotic competes with FAD. The dissociation constant of the complex of this antibiotic with the protein in competition with FAD was calculated to be $1.8 \cdot 10^{-2}$ *M*.

Using FMS and AMS as indicators, v_0/v plotted against the concentration of streptomycin gave a curve in the presence of FMS and a straight line in the presence of AMS, respectively, as shown in Fig. 2. From these results, it may be concluded that streptomycin also inhibits the oxidase activity in competition with the AMP part of FAD.

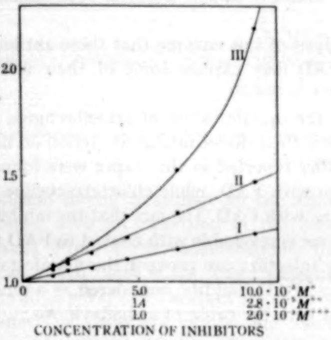

Fig. 2. Analysis of the inhibitory action of streptomycin on D-amino acid oxidase, using kinetic indicators. v_0 and v represent the reaction velocities in the absence and presence of inhibitors. The reaction mixture contained 15 μg of the oxidase protein, 0.15 M of DL-alanine and $1.7 \cdot 10^{-7}$ M of FAD.
* streptomycin concn., ** FMS concn., *** AMS concn. Curve I: in the presence of streptomycin. Curve II: in the presence of streptomycin and AMS. Curve III: in the presence of streptomycin and FMS. V was 132 μl oxygen uptake/30 min and v_0 was 80 μl oxygen uptake/30 min.

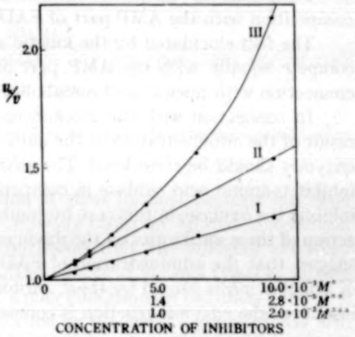

Fig. 3. Analysis of the inhibitory action of penicillin on D-amino acid oxidase, using kinetic indicators. v_0 and v represent the reaction velocities in the absence and presence of inhibitors. The reaction mixture contained 15 μg of the oxidase protein, 0.15 M of DL-alanine and $1.7 \cdot 10^{-7}$ M of FAD.
* penicillin concn., ** FMS concn., *** AMS concn. Curve I: in the presence of penicillin. Curve II: in the presence of penicillin and AMS. Curve III: in the presence of penicillin and FMS. V was 132 μl oxygen uptake/30 min and v_0 was 80 μl oxygen uptake/30 min.

Inhibitory action of penicillin

Using Methods I and II, penicillin was also found to inhibit the oxidase activity in competition with FAD. Since no complex formation between penicillin and FAD could be detected by spectrophotometry and fluorimetry, the dissociation constant of penicillin with the protein in competition with FAD was calculated to be $1.4 \cdot 10^{-2}$ M.

The kinetic analysis using FMS and AMS gave the same result as for chloramphenicol and streptomycin as shown in Fig. 3.

DISCUSSION

Chloramphenicol was found to inhibit D-amino acid oxidase by a single mechanism connected with FAD. The fact that chloramphenicol quenches the fluorescence of FAD indicates some interaction of these compounds in aqueous solution. However, the fact that the inhibition of the enzyme by chloramphenicol is a single mechanism and that it competes with the AMP part of FAD suggests that there can be no complex formation between this antibiotic and the isoalloxazine nucleus. The absorption spectrum obtained lends further support to this view. Consequently, the quenching action of chloramphenicol on the fluorescence of FAD may be attributed to its second type of collision.

Streptomycin and penicillin were also found to inhibit D-amino acid oxidase in competition with the AMP part of FAD.

The fact elucidated by the kinetic analysis of this enzyme that these antibiotics compete equally with the AMP part of FAD may explain some of their roles in connection with nucleic acid metabolism.

In connection with the mechanism of the manifestation of ariboflavinosis as a result of the administration of the antibiotics, their direct inhibitory action on flavin enzymes should be considered. The antibiotics reported in this paper were found to inhibit D-amino acid oxidase in competition with FAD, while chlortetracycline also inhibits the oxidase, in this case by combining with FAD. The fact that the inhibitory action of these antibiotics on the flavin enzyme is reversible with respect to FAD may suggest that the administration of FAD by injection can prevent the manifestation of ariboflavinosis caused by these antibiotics: FAD could be considered as a remedy insofar as the enzymic reaction is connected with the cause of ariboflavinosis.

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ENCOUNTER EFFICIENCY OF COLIPHAGE-BACTERIUM INTERACTION

ARTHUR L. KOCH

Department of Biochemistry, College of Medicine, University of Florida, Gainesville, Fla. (U.S.A.)

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SUMMARY

A collision theory treatment for the adsorption of virus by host bacteria has been combined with an extension of the SMOLUCHOWSKI diffusion limited coagulation equation. From these considerations, the conclusion that the collision efficiency may approach unity is not found warranted. The effects of various types of mixing on the adsorption rate constant are discussed. Under conditions of diffusion limitation the effects of Brownian motion and sedimentation in the earth's gravitational field are considered and shown to be small though significant. More vigorous mixing is produced by laboratory aeration procedures and results in an increase in the diffusion limited rate.

INTRODUCTION

Since the fundamental papers of SCHLESINGER[1] and DELBRÜCK[2] the adsorption of virus onto its host bacterium has been interpreted in terms of the SMOLUCHOWSKI coagulation equation. SMOLUCHOWSKI's derivation[3] is based on the assumption that diffusion of one reactant toward the other is the rate limiting process. As such it does not depend on the encounter efficiency; *i.e.*, the probability, P, that a collision will result in reaction or infection. None the less, it has commonly been assumed that agreement between the rate constant for virus adsorption obtained under optimal conditions with the calculated rate is evidence for high encounter efficiency. That this does not follow is shown below.

FICK's second law takes the following form under conditions of radial symmetry:

$$\frac{\partial C}{\partial t} = \frac{D}{r} \frac{\partial^2(rC)}{\partial r^2} \tag{1}$$

where r is the radial distance; t, the time; D, the diffusion constant and C, the concentration of the diffusing substance.

For spherical particles of radius r_0, SMOLUCHOWSKI solved this equation with the initial condition that a uniform concentration C_∞, existed at all regions external to the particle. In addition it was assumed that the concentration at the edge becomes and remains zero because of reaction at the surface. His solution is as follows:

$$C = C_\infty \left[1 - \frac{r_0}{r} + \frac{2r_0}{r\sqrt{\pi}} \int_0^{(r-r_0)/2\sqrt{Dt}} e^{-\omega^2} \quad d\omega \right] \tag{2}$$

The rate of uptake per particle, dq/dt, can then be obtained from FICK's first law:

$$\frac{\mathrm{d}q}{\mathrm{d}t} = 4\pi r_0^2 D \left(\frac{\partial C}{\partial r}\right)_{r=r_0} = 4\pi D r_0 C_\infty \left[1 + \frac{r_0}{\sqrt{\pi D t}}\right] \tag{3}$$

In general, the transient term becomes negligible after a very short time and may be disregarded. Thus, we obtain for the rate constant, $k_{\max}$, the familiar expression:

$$k_{\max} = \frac{\mathrm{d}q}{\mathrm{d}t} \cdot \frac{1}{C_\infty} = 4\pi D r_0 \tag{4}$$

But as HERSHEY[4] has pointed out, this relationship must fail completely as a measure of number of collisions under conditions in which k is less than maximal. Under such conditions the concentration at the edge of the particle will no longer be zero, and many encounters will ensue before a fruitful one takes place.

We must therefore solve equation (1) under less rigid boundary conditions than those assumed by SMOLUCHOWSKI. This can be done very simply for the steady-state case by setting dC/dt = 0. The solution can be written:

$$C = C_\infty \left[1 - \frac{a}{r}\right] \tag{5}$$

This equation differs from equation (2) in that the integral term has vanished because of the steady state assumption, and, moreover, r_0 has been replaced by an arbitrary parameter, a. This equation is generally applicable to many cases of cell metabolism, see RASHEVESKY[5]. For our present case in which the diffusing substance is only utilized at the edge of the particle $0 \leq a \leq r_0$ and, therefore, the concentration at the surface of the particle, C_0, is given by:

$$0 < C_0 = C_\infty \left[1 - \frac{a}{r_0}\right] \leq C_\infty. \tag{6}$$

The rate of uptake may be calculated as before:

$$\frac{\mathrm{d}q}{\mathrm{d}t} = 4\pi r^2 D \left(\frac{\partial C}{\partial r}\right) = 4\pi r^2 D C_\infty \left(\frac{a}{r^2}\right) = 4\pi D a C_\infty \tag{7}$$

and the corresponding rate constant becomes:

$$k = 4\pi D a. \tag{8}$$

Now a is a parameter that will be only equal to the physical radius of the particle if the rate of the reaction is sufficient to reduce C_0 to zero. This equation is more generally applicable than is equation (4) and is the equation employed by DELBRÜCK[2].

A more appropriate estimate of the number of collisions derives from classical collision theory[6]. The suitable expression for the rate of collision of small particles with a single large particle is then:

$$z = C_0 r_0^2 \sqrt{\frac{8\pi RT}{m}}, \tag{9}$$

where R is the gas constant $8.3144 \cdot 10^7$ g cm^2 sec^{-2} deg.$^{-1}$; T, the absolute temperature; and m, the mass of the small particle. The arguments to justify the application of this formula to large particles such as virus and bacteria are precisely those employed to justify the use of the MAXWELL-BOLTZMAN distribution to the particles used in the study of Brownian motion[7]*.

Upon eliminating C_0 between equation (6) and equation (9) we obtain:

$$z = C_\infty \left[r_0^2 \sqrt{\frac{8\pi RT}{m}} \right] \frac{r_0 - a}{r_0} : a \neq r_0. \qquad (10)$$

The factor in the brackets will be designated k_c and is the quantity comparable to the rate constant $k_{\max}$ of the SMOLUCHOWSKI treatment. It is the specific rate constant for the collision process in the absence of reaction.

The encounter efficiency may now be calculated from the quotient of the rate of uptake, dq/dt, by the rate of collision, z.

$$P = \frac{1}{z}\frac{dq}{dt} = \frac{4\pi DaC_\infty}{C_\infty r_0^2 \sqrt{\frac{8\pi RT}{m}} \frac{r_0 - a}{r_0}} = \frac{k_{\max}}{k_c} \frac{a}{r_0 - a} : a \neq r_0 \qquad (11)$$

Let us now calculate $k_{\max}$ and k_c assuming: (a) $r_0 = 0.8 \cdot 10^{-4}$ cm the radius of a sphere of the same surface area as the bacteria actually possesses[9]**, (b) $T = 310.16°$ K, (c) $D = 4.0 \cdot 10^{-8}$ cm^2/sec at 20° for T_6,[11] (d) $m = 167 \cdot 10^6$ (ref. 12). In addition the STOKES-EINSTEIN relation must be employed to correct the diffusion constant to 37° and to the viscosity of synthetic glucose medium. With these constants $k_{\max}$ is found to be $3.58 \cdot 10^{-9}$ cm^3 min^{-1}; and k_c, $2.39 \cdot 10^{-5}$ cm^3 min^{-1}. Substituting these values into equation (11) we obtain:

$$P = 1.5 \cdot 10^{-4} \left(\frac{a}{r_0 - a} \right) : a \neq r_0 \qquad (12)$$

A plot of this equation is shown in Fig. 1. It can be seen that a decrease of a factor of two from the maximum rate changes P from unity to $1.5 \cdot 10^{-4}$. Thus, at high adsorption rates tremendous precision both experimentally and with regard to refined diffusion models would be required to make even the crudest estimate of P.

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[*] The assumption made in the derivation of the general equation, equation (15), is that the rate of adsorption is proportional to the surface concentration. The conditions under which this is valid have been discussed by COLLINS AND KIMBALL[8], who conclude that the assumption is valid for diffusion for the microscopic or Brownian motion case as it is for the macroscopic case. Viewed as a problem in diffusion, no physical significance would be attached to the product Pk_c. Yet equation (15) applies for very small r_0 where P clearly has the significance of a collision efficiency and k_c the significance of the specific collision number. These considerations add further support to application of collision theory to large adsorbing particles.

[**] There is no theoretical justification for this assumption. Alternatively, and with more justification, we could assume the bacterium to be a long thin rod. In this case (see ref. 10, p. 282) the rate of adsorption changes continuously (*e.g.*, no constant steady state is achieved). However, the rate changes very slowly after a short time interval. Since Brownian motion and settling cause effective mixing in times of the order of seconds, we may readily calculate the adsorption rate constant of a thin rod of length, l, one second after it remains in a given position. The calculation shows that $k_{\max}$ is of the order of two thirds of $4\pi Dl$, and is independent of the radius of the rod. Thus, instead of the radius of the sphere of the same surface area, a larger value approaching two thirds the length of the rod should be employed. For *E. coli*, these two approaches lead to values for $k_{\max}$ differing by about 65%.

The absence of appreciable dependence of observed rate on P probably accounts for the success that has attended the application of the SMOLUCHOWSKI equation to the coagulation of colloids[7, 13].

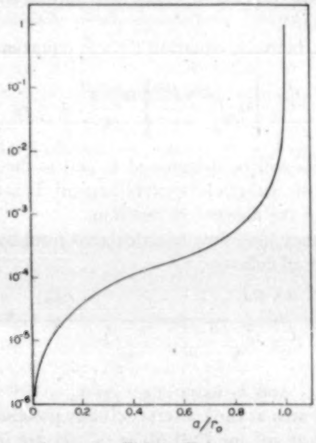

Fig. 1. Dependence of collision efficiency upon particle radius.

If equation (11) is solved for a, and this value substituted into equation (8), the following expression for k is obtained:

$$k = \frac{(4\pi D r_0) \cdot Pk_e}{4\pi D r_0 + Pk_e} \text{ or } \frac{k_{\max} \cdot Pk_e}{k_{\max} + Pk_e}. \tag{13}$$

This relationship shows that the apparent adsorption rate constant will depend only on D and r_0 if $Pk_e > 4\pi D r_0$ and only on P and k_e if the reverse is true.

Turning now to another aspect of the problem, let us consider the effects of mixing on the adsorption rate. The SMOLUCHOWSKI treatment idealizes the actual situation in two important points. In the first place we have seen that the concentration at the surface need not become zero, and in the second place, the adsorption rate constant at time zero is not infinite but is Pk_e.

This should also be the extrapolated value for the rate constant under conditions of greater and greater stirring. (Under conditions of violent stirring, other phenomena come into play, see below.) When P is low, and consequently, $k < k_{\max}$, k will not be increased by moderate stirring. When k is of the order of $k_{\max}$, the effects of stirring become large, since then P must be large and

$$k_{\text{stir}} = Pk_e = \frac{k \cdot k_{\max}}{k_{\max} - k} : k \neq k_{\max} \tag{14}$$

where k and k_{stir} are the adsorption rate constants in the unstirred and stirred cases respectively. Such experiments in addition to yielding information about P would also yield an accurate value for $k_{\max}$ in regions in which both diffusion and collision are limiting.

For the quantitative evaluation of the effects of stirring, the complete solution of the diffusion problem under more generalized boundary conditions is required. This solution[8,10]*, in terms of the quantities defined here is:

$$k = \frac{k_{\max}\quad Pk_e}{k_{\max} + Pk_e}\left[1 + \frac{Pk_e\,e^{+\theta^2}}{k_{\max}}\frac{2}{\sqrt{\pi}}\int_{\theta}^{\infty} e^{-\omega^2}\,d\omega\right], \tag{15}$$

where

$$\theta = \frac{\sqrt{Dt}}{r_0}\,\frac{Pk_e + k_{\max}}{k_{\max}}.$$

It is readily seen that this equation reduces to equation (13) when t is large. At very small values of time, it reduces to $k = Pk_e$. In like manner, equations (3), (4), (8), (9), and (10) can be derived as limiting cases from this equation.

Under conditions where P is high, and consequently,

$$\frac{k_{\max}\,r_0}{k_{\max} + Pk_e}$$

is small and thus, adsorption is essentially, but not necessarily, completely diffusion limited, the equation simplifies to:

$$\frac{dq}{dt} = 4\pi DaC_\infty\left(1 + \frac{r_0}{\sqrt{\pi Dt}}\right) \tag{16}$$

This equation predicts a faster rate of adsorption initially and also a more rapid decrease to the steady state rate than may actually be the case. For relatively slow mixing processes these two effects should be largely compensatory, and therefore, independent of the value of Pk_e. The average rate that would be observed if the bacterium remained stationary with respect to its environment for a length of time, and then is moved discontinuously to a new position where the concentration is C_∞ can then be calculated:

$$\overline{\frac{dq}{dt}} = \frac{\int_0^\infty \frac{dq}{dt}\,dt}{\tau} = \frac{4\pi DaC_\infty}{\tau}\left(\tau + \frac{2r_0\sqrt{\tau}}{\sqrt{\pi D}}\right) = 4\pi Da\,C_\infty\left(1 + \frac{2r_0}{\sqrt{\pi D\tau}}\right) \tag{17}$$

The increase in the observed rate constant will be $2r_0/\sqrt{\tau\pi D}$. The effect of mixing will then be of this order of magnitude, if τ is taken to be the minimum time required to move to a region of practically uniform virus concentration. This distance is of the order of twice the radius of the bacterium since there the concentration must be at least two thirds of the bulk concentration. For Brownian motion** an increase in $k_{\max}$ of 25 % can be calculated. For motion due to gravity alone, an increase of about 22 % is calculated. The combination of these two motions would give little further increase over either alone.

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* See footnote page 313.

** It should be noted that this is a different expression than is ordinarily assumed for reactions due to combined Brownian motion of the particles[7,14]. It is ordinarily assumed that the effective diffusion constant is the sum of the diffusion constants of the two reactants, $D + D_b$, but equation (17) shows that it is of the order of $D + \sqrt{2DD_b/\pi}$, where D_b is the diffusion constant of the bacterium. This formula predicts a much greater influence of the diffusion of the bacterium than is predicted by the sum of the two diffusion constants. This is because the bacterium diffuses out of the gradient well it has produced. If the diffusion constant D_b were of the order of D, this expression approximates the sum of the diffusion constants of the two reactants. The slight deviation no doubt results from the approximation of the treatment. The calculation could be improved by taking τ equal to the time to go $\sqrt{8/\pi}$ the radius instead of the time required to move twice the radius of adsorbing particle.

For motile organisms, the effect can be much larger. *Vibrio cholerae* can move 50 times its own diameter in a second. The maximum rate that this organism could remove suitable virus (assuming a diffusion constant of $4 \cdot 10^{-8}$ cm^2/sec) is 320 % greater than when the organism is at rest.

The motility of *Vibrio cholerae* is exceptional and most motile organisms move ten fold slower. One can, therefore, expect that a motile *Escherichia coli* should adsorb virus about 100 % faster than the non-motile forms.

DELBRÜCK[2] suggested that motility of the organisms under the special conditions of early broth culture would account for the high value of the observed adsorption rate constant. The increases he observed between the young cultures compared to older cultures were, however, much larger than the two fold increase calculated here. Therefore, some alternative explanation must be sought for his results.

Returning to the effects of mechanical mixing, it may be noted that in order to observe an effect under diffusion limited conditions, it is necessary to stir in such a way that the stirring is effective at the microscopic level as well as the macroscopic level. Although fluid dynamics is a well advanced study, questions as to the microscopic mixing or shear in turbulent flow have not received adequate consideration. In the case of laminar flow through a capillary, the shear is greatest at the edge and decreases to zero at the axis. Average shears of the order of 10^2/sec to 10^3/sec can be produced in ordinary laboratory viscometers. This means that a point in the solution 1 μ removed from a second point in the direction normal to flow is moving 100 to 1000 μ/sec on the average with respect to the second point. Thus, the bacterium is displaced to bulk environment at 0.001 to 0.01 sec intervals. This should have the influence of increasing the adsorption rate by 4.5 to 14.0 times. These factors are the appropriate averages computed for the variations in shear across the capillary.

Finally, we wish to consider the influence of stirring produced by aeration of a culture medium. Aeration is standard in bacteriology, and is routine for the production of phage lysates In fact, vigorous aeration is usually used when high titer lysates are to be prepared.

The shear produced by the ascent of a small air bubble in a liquid may be calculated from the STOKES' equation and from the considerations involved in its derivation[15]. The shear, $(\mathrm{d}u/\mathrm{d}r)_{\perp}$, perpendicular to the motion of the sphere may be written:

$$\left(\frac{\mathrm{d}u}{\mathrm{d}r}\right)_{\perp} = \frac{1}{6}\,\frac{r_0}{\eta}\left(\frac{r_0^2}{r^2}\right)\left(1 + \frac{r_0^2}{r^2}\right)(\sigma - \rho)\, g \qquad (18)$$

where σ and ρ are the density of the medium and of the bubble, η, the viscosity of the medium and g is the acceleration due to gravity. Evaluation of this expression for an air bubble 1 mm in diameter shows that a shear of 260/sec is produced at a distance of 0.5 mm from the surface of the sphere. Significant shears are produced out to distances 10 times the radius. The shear is not uniform in all directions from the sphere. However, we can assume that the air bubble will cause shears that will be important (> 10/sec) in a volume of the order of 1000 times that of the air bubble. Since the amount of air flowing at any time through the solution is usually more than 0.001 of the liquid volume, we can expect that the effects of this stirring will strongly influence the observed adsorption rate.

Finally, the effects of violent stirring will be considered. The actual number of collisions is increased by stirring. The collision number due to this effect was derived by SMOLUCHOWSKI and by TUORILA (see OVERBEEK[14]). The expression is:

$$z = \frac{4}{3} C_{\infty} r_0^3 \left(\frac{\mathrm{d}u}{\mathrm{d}r}\right) \tag{19}$$

Since this expression depends on the cube of the radius, the effect is insignificant for small particles. For an object the size of *E. coli* shears of the order of 10^5/sec are necessary to cause an increase in the number of collisions above that predicted by equation (9). Therefore, only in exceptional conditions will the process be important.

CONCLUSIONS

These considerations show that information regarding the probability of collision of virus with its host bacterium can not be assessed by existing experimental data. However, since variations in ionic strength[16] and tryptophan concentration[17] change the adsorption rate constant smoothly, though rapidly, from regions in which P is obviously small to regions in which diffusion is limiting, it appears that the P is probably small at least throughout much of the region in which the adsorption rate appears to be experimental maximal. Other reasons for assuming P to be small have been given by WEIDEL[18] and by HERSHEY[4].

We have not considered the cage effect[19] which will result in clusters of collision events. This effect would have the influence of raising P per cluster, but not of influencing P per individual collision.

These considerations suggest that P might be measured by its dependence on shear, realizing that as shear is increased from zero, there will be a region in which equation (14) will have validity but that this relationship will break down at higher values of shear, $> 10^5$/sec. In actual fact, at high values of shear, no adsorption will take place as shown by the experiments of ANDERSON[20], presumably because the union can not last long enough. However, restricting consideration to values of shear that do not interfere with adsorption, it should be possible to obtain reliable estimates of P under conditions where P has a value less than 10^{-3}. On the other hand, it should be possible to calculate $k_{\max}$ from experimental data if P is greater than 10^{-5}. $k_{\max}$ calculated in this way would contain the effects of Brownian motion and settling when P is large, but not when P is small.

Finally, we should note that P as used here includes not only steric factors, but an Arrhenius activation term, and electrostatic and entropy terms.

ACKNOWLEDGEMENT

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A STABLE RIBONUCLEOPROTEIN FOR AMINO ACID INCORPORATION

MITURU TAKANAMI

National Institute of Animal Health, Kokobunji, Tokyo (Japan)

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SUMMARY

A ribonucleoprotein (RN-P), which is stable and active for incorporation *in vitro* of [^{14}C]amino acid, has been prepared from rat liver in an almost pure form. The method of purification is based on the fact that the RN-P complex is reversibly precipitated by an increasing concentration of divalent cations, such as Mg and Ca. In the purified preparation, the ratio of u.v. absorption maximum (260 mμ) to minimum (235 mμ) was 1.58 and the N:P ratio approximately 3.34. Maximum incorporation *in vitro* of [^{14}C]amino acid into the purified RN-P was obtained by addition of ATP, an ATP-generating system, GTP, and a soluble protein fraction. The optimal pH for incorporation was in the range of 7.8–8.0, and the reaction was accelerated by a low concentration of KCl.

INTRODUCTION

Since the important role of the microsomal fraction in protein synthesis of the cell has been emphasized[7,8,11,13,14,19], it becomes important to study the physicochemical properties of RN-P purified from the microsomal fraction and the requirements for

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Abbreviations: RN-P, ribonucleoprotein; RNA, ribonucleic acid; RNAase, ribonuclease; ATP, GTP, CTP, UTP, triphosphates of adenosine, guanosine, cytidine, and uridine; Tris, tris-(hydroxymethyl)aminomethane; EDTA, ethylenediamine-tetraacetate.

incorporation *in vitro* of amino acids into the purified RN-P. However, purification of RN-P from animal tissues is made difficult because of the extreme instability of the complex and the contamination with metalloprotein[10, 16].

In the course of investigation of the properties of the RN-P complex prepared from animal tissues, it was found that the RN-P was reversibly precipitated in an increasing concentration of divalent cations, such as Mg and Ca, and further it proved possible to obtain RN-P in almost pure form.

This paper deals with the method of purification of RN-P from rat liver, some physicochemical properties of the purified RN-P, and the requirements for incorporation *in vitro* of [^{14}C]leucine into the purified RN-P.

MATERIALS AND METHODS

Preparation of crude ribonucleoprotein fraction

The preparation of crude RN-P fraction from the liver homogenate was essentially the same as already reported[2, 7, 10]. Livers harvested from male rats fasted overnight were minced and suspended in ice-cold buffer medium to prepare about 15 % homogenate. The buffer was composed of 0.12 *M* KCl, 0.005 *M* $MgCl_2$, and 0.02 *M* potassium phosphate buffer pH 7.6 (ref. 16). After homogenation in a Waring blender for 3 min at a low speed, the pH was adjusted to about 7.6. Then the homogenate was spun for 30 min at 44,000 × *g* and the resulting supernatant was centrifuged for 2 h at 105,000 × *g*[16]. The jelly-like pellet obtained was resuspended in 0.001 *M* $MgCl_2$ containing 0.002 *M* phosphate buffer pH 7.8, and was dissolved completely by addition of Na-deoxycholate to a concentration of 0.6–0.8 %. The suspension was centrifuged for 30 min at 44,000 × *g* and the resulting supernatant for 2 h at 105,000 × *g*. The final pellet contained most RN-P, and the suspension in 0.001 *M* $MgCl_2$ had an ultraviolet absorption maximum at 260 mμ and a minimum at 240 mμ. However, the solution still had a yellow colour owing to the contamination by metalloprotein, and its ratio of ultraviolet absorption maximum to minimum was lower than 1.3 (Fig. 1).

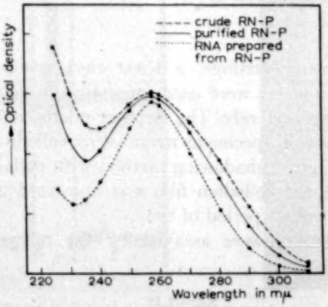

Fig. 1. U.v. absorption spectrum of the purified ribonucleoprotein (RN-P).

Further purification of ribonucleoprotein

The purification of the crude RN-P fraction was performed, taking advantage of the fact that RN-P is reversibly precipitated by an increasing concentration of

Mg ion. When $MgCl_2$ was added to a concentration of 0.05 M, RN-P precipitated completely. The precipitate was separated by low-speed centrifugation, washed once with 0.01 M $MgCl_2$, resuspended in distilled water, and dialysed to remove excess Mg ions. Small amounts of insoluble materials were separated by centrifugation for 20 min at about 13,000 × g. The solution obtained was colourless and quite clear. By this procedure, the ratio of u.v. absorption maximum to minimum was increased from 1.3 to approximately 1.6 (Fig. 1) and at the same time the N:P ratio of the preparation changed to 3.3–3.4; for the crude RN-P preparation the latter ratio was 4.9. The purified RN-P was stored in a deep freezer or freeze-dried.

Technique for incorporation in vitro *of* [^{14}C]*leucine*

The technique used for incorporation *in vitro* was based, in principle, on that of LITTLEFIELD AND KELLER[8] and SACHS[11]. The basal medium for incorporation was composed of 0.06 M KCl, 0.005 M $MgCl_2$, and 0.05 M Tris buffer pH 7.8. To this medium the purified RN-P, the pH 5 enzymes fraction, ATP, GTP, phosphocreatine, and ATP-creatine transphosphorylase were added so as to bring the total volume in a tube to 1.0 ml. The tube was then incubated at 37° in air with ^{14}C-L-leucine uniformly labelled. ATP, GTP, and phosphocreatine used were sodium salts of Sigma Chemical Co. and the ATP-creatine transphosphorylase was prepared from rabbit muscle by the method of KUBY *et al.*[6]. The pH 5 enzymes fraction was prepared from the supernatant obtained by centrifugation at 105,000 × g for 2 h as described by HOAGLAND *et al.*[3,4].

After incubation, the reaction was stopped by addition of cold 0.5 N perchloric acid and treated with 1 N NaOH for 1 h at 37°. After neutralization with 1 N perchloric acid, the precipitate was washed twice with cold 0.2 N perchloric acid, and mounted on a filter paper fixed to a glass filter. After drying, it was counted under the low background gas-flow counter (background count is lower than 1 count/min). By the above procedure, the value for zero-time became lower than 3 count/min. All the values obtained by counting were corrected with regard to protein concentration, using the self-absorption curve of protein.

Physical and chemical analysis

A Spinco model E ultracentrifuge, a Klett electrophoresis apparatus, and a Beckman DU spectrophotometer were used for sedimentation, electrophoresis, and u.v. absorption analysis, respectively. The electron microscope used was of Hitachi HU-9 type and observations on specimens mounted on collodion film were performed by two different methods, *viz.* a shadowing method with palladium and an electron-staining method in which the collodion film was immersed in a dilute solution of phosphotungstic acid for a short period of time.

Nitrogen and phosphorus were assayed by the micro-kjeldahl and Allen's method.

RESULTS

Properties of purified ribonucleoprotein

The purified RN-P preparation gave an u.v. absorption maximum at 260 mμ and a minimum at 235 mμ owing to its high RNA content as shown in Fig. 1. The chemical and physical data of the preparation are summarized in Table I.

TABLE I

CHEMICAL AND PHYSICAL DATA ON PURIFIED RIBONUCLEOPROTEIN FROM RAT LIVER

NP ratio	3.3_4
U.v. absorption maximum	260 mμ
minimum	235 mμ
260/235 mμ ratio	1.5_8
Sedimentation coefficient s_{20°	79, 39, 58, 116
Electrophoretic mobility μ_{0°	$9.7 \cdot 10^{-5}$ cm²/sec. V

The sedimentation pattern of the RN-P preparation gave four peaks of which the main peak was at about 70–80 S, showing considerable sharpness (Fig. 2a). Extrapolated values of the four peaks for infinite dilution were 39 S, 58 S, 79 S and 116 S, respectively. However, it was noticed that the quantitative relationships among the four peaks varied somewhat according to the medium employed, and particularly according to the concentration of Mg ion. A slight increase of Mg ion concentration caused the rise of the 116 S peak (Fig. 2b). A decrease of Mg ion concentration by dialysis in distilled water or by treatment with EDTA in low concentration on the other hand, caused the rise of the 39-S and 58-S peaks, with a corresponding decrease in the 79-S component (Fig. 2c). When the Mg ion was removed by a high EDTA concentration, a main peak appeared at the 39-S unit (Fig. 2d). Such changes were reversed by addition of Mg or Ca ion, and when $MgCl_2$ or $CaCl_2$ was added in a concentration of 0.02 *M* and the RN-P precipitated was resuspended in distilled water, the sedimentation pattern revealed the main peak at 79 S. It was therefore concluded that the components faster than 39 S were aggregates of the 39-S unit and that the 79 S unit was a relatively stable aggregate. However, minor components slower than 25 S in sedimentation velocity appeared on

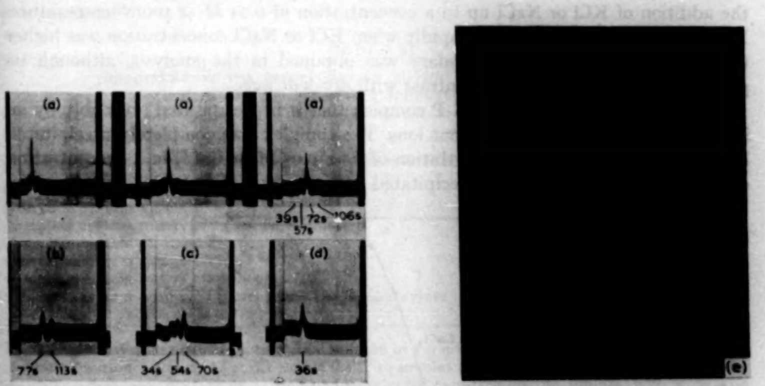

Fig. 2. Sedimentation patterns (a-d) and electron micrograph(e) of the purified ribonucleoprotein (RN-P). (a) RN-P in 10^{-3} *M* $MgCl_2$, taken at 4 min intervals after reaching a speed of 30,000 rev./min. (b) RN-P in $5 \cdot 10^{-3}$ *M* $MgCl_2$, 12 min after reaching speed of 30,000 rev./min. (c) RN-P dialysed against distilled water for 20 h at 4°, 16 min after reaching a speed of 30,000 rev./min. (d) RN-P treated with 0.02 *M* EDTA at 20° for 30 min, 16 min after reaching speed of 30,000 rev./min. (e) Electron micrograph taken at a magnification of 20,000 ×, shadowed at an angle of about 20° with palladium.

dialysis or treatment with EDTA (as shown near the meniscus in the photographs of Fig. 2c, d). Such components are considered to be degradation products of the RN-P, because the RN-P complex was extremely unstable in a Mg-free medium[1,17]. In this connection, the sedimentation pattern of RNA prepared from the RN-P by the use of phenol[5] gave three peaks at approximately 3 S, 17 S, and 23 S.

Electron-microscopically, the preparation consisted of almost spherical and uniform particles, as shown in the electron micrograph of Fig. 2e. The size of particles measured by the electron-staining method was approximately 21 mμ. This value coincided with that estimated from the sedimentation data, according to which, from the composition of RNA, the 79 S component is estimated to be about 22 mμ in diameter, assuming that the shape is spherical and the hydration about 30%. However, particles a little more than 10 mμ in size were also observed in the field and seemed to correspond to the smaller units appearing in the sedimentation analysis.

Electrophoretic analysis of the RN-P showed considerable homogeneity and its mobility was recorded as $9.7 \cdot 10^{-5}$ cm^2/sec. V, when the analysis was carried out at 4° in 0.1 *M* Tris buffer pH 7.4.

The purified RN-P was considerably stable when dissolved in 10^{-3} *M* $MgCl_2$, and remained unchanged for a few months in a deep freezer or after freeze-drying, whereas the crude RN-P fraction was unstable on freezing and readily caused flocculation owing to the dissociation of the complex. Particularly, it was noted that freeze-dried RN-P showed an ultracentrifugal pattern similar to that of the original preparation.

The purified RN-P, in 10^{-3} *M* $MgCl_2$, was affected only very slightly by the shift of pH and was stable within a pH range between 6 and 9, as determined by centrifugal runs. At the same time, the sedimentation pattern was not greatly influenced by the addition of KCl or NaCl up to a concentration of 0.24 *M* at room temperature. However, aggregation occurred rapidly when KCl or NaCl concentration was higher than 0.5 *M*, and spreading boundary was obtained in the analysis, although no precipitation was produced, in contrast with divalent ions.

It is characteristic of the RN-P complex that it is precipitated reversibly by an increasing concentration of divalent ions. The complex was completely precipitated by addition of $MgCl_2$ in a concentration of 0.02–0.05 *M* or $CaCl_2$ in a concentration of 0.01–0.02 *M* (Fig. 3). The precipitated complex was dissolved readily by the

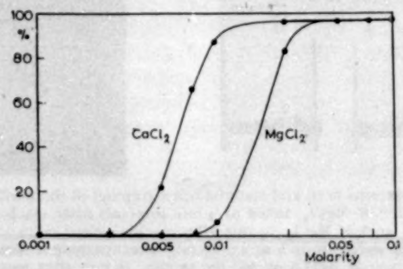

Fig. 3. Reversible precipitation of the ribonucleoprotein complex by divalent cations. The longitudinal scale shows percentage of the precipitated portion to the original solution.

reduction of such cations and showed a sedimentation pattern similar to that of the original solution. In this connection, such curves of precipitation were very similar to those of RNA prepared from RN-P by the use of phenol[5], and it will be indicated that the property of the RN-P complex is greatly influenced by that of RNA.

When the RN-P suspension was treated with 10 to 50 μg per ml of RNAase (Worthington B.C.), 89–92 % of the RNA of the original complex was degraded to an acid-soluble fraction within 60 min at 37°. At the same time, the protein fraction gave a flocculent precipitation. So the whole protein and about 8 % of the RNA of the original complex was sedimentable by low-speed centrifugation. The protein aggregate was insoluble in strong salt solution, in urea, and in acid, but dissolved in a solution with a pH higher than 9.0, as if it were a denatured protein.

Incorporation in vitro *of* [^{14}C]*leucine into ribonucleoprotein*

When the purified RN-P was incubated with L-[^{14}C]leucine, ATP, and ATP-generating system, GTP, and the pH 5 enzymes fraction, rapid incorporation of [^{14}C]-leucine into RN-P took place and omission of any factor from the incubation system stopped or lowered the incorporation (Table II). However, considerable activity occurred in the absence of ATP-creatine transphosphorylase, perhaps because the pH 5 enzyme fraction contained some transphosphorylation activity[8]. On the other hand, the addition of such agents as CTP, UTP, SH-compounds (*e.g.*, glutathione and cysteine), cell dialysate (obtained from liver homogenate), and yeast extract did not increase the activity. Nor was any stimulation obtained by adding a mixture of seventeen L-amino acids (each 0.1 μM) excluding leucine. Substitution of the whole soluble fraction for the pH 5 enzymes fraction did not increase the activity. It was therefore certain that the factors stated above were essential for the incorporation, as was shown in experiments with whole liver microsome[8,11,19] and with RN-P from pea seedlings[18].

TABLE II

REQUIREMENTS FOR INCORPORATION *in vitro* OF L-[^{14}C]LEUCINE INTO THE PROTEIN OF PURIFIED RIBONUCLEOPROTEIN (RN-P)

Components	*Protein (counts/min/mg)*
Complete system	169
Complete system minus RN-P	7
Complete system minus pH 5 enzymes fraction	26
Complete system minus ATP	19
Complete system minus GTP	77
Complete system minus phosphocreatine	35
Complete system minus ATP creatine transphosphorylase	112

The complete system contained, in a final volume of 1.0 ml per tube, 1.2 mg RN-P, 2.0 mg pH 5 enzymes fraction, 1 μmole ATP, 0.1 μmole GTP, 10 μmoles phosphocreatine, 0.1 mg ATP-creatine transphosphorylase, 0.1 μmole L-[^{14}C]leucine ($2.2 \cdot 10^5$ counts/min), 60 μmoles KCl, 5 μmoles $MgCl_2$, and 50 μmoles Tris buffer of pH 7.8. Incubation was done for 20 min at 37°.

Such incorporation continued for 40 min, as illustrated in Fig. 4, while the speed of incorporation dropped with the advance in incubation and the rate of it varied with the quantity of the pH 5 enzymes fraction. The activity of incorporation was not influenced by freeze-drying of RN-P, in contrast with crude RN-P fraction, which rapidly lost the activity when subjected to freezing. The requirements for the maximum incorporation of freeze-dried RN-P were the same as in the case of a fresh preparation. The reaction was inhibited completely by the pretreatment of both RN-P and the pH 5 enzymes fraction with RNAase (10 μg per ml, for 30 min at 37°) or by the addition of 10 μg of RNAase to the incubation mixture, as with the system of whole microsome–pH 5 enzyme fraction[8,9].

When the effect of the concentration of each factor required for incorporation was examined, the optimal concentration was obtained with regard to ATP and phosphocreatine and the saturation concentration with regard to GTP and ATP-creatine transphosphorylase (Fig. 5). There was also an optimal concentration for neutral salts, as illustrated in Fig. 6. However, KCl accelerated the reaction two or three times as much as NaCl. Optimal pH for the incorporation was on the slightly alkaline side (Fig. 6).

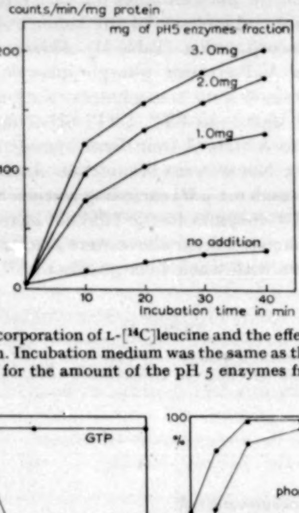

Fig. 4. Time curves of the incorporation of L-[^{14}C]leucine and the effect of varying the concentration of the pH 5 enzymes fraction. Incubation medium was the same as the complete system in Table II, except for the amount of the pH 5 enzymes fraction.

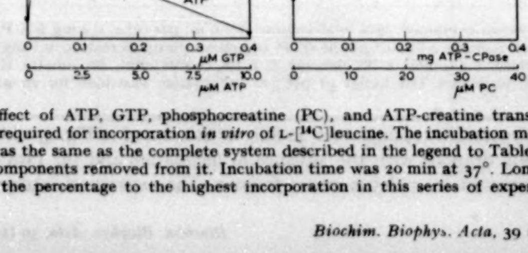

Fig. 5. The effect of ATP, GTP, phosphocreatine (PC), and ATP-creatine transphosphorylase (ATP-CPase) required for incorporation *in vitro* of L-[^{14}C]leucine. The incubation medium for each experiment was the same as the complete system described in the legend to Table II, except for the various components removed from it. Incubation time was 20 min at 37°. Longitudinal scale shows the percentage to the highest incorporation in this series of experiments.

From these experiments, it is concluded that the full activity for incorporation *in vitro* of [^{14}C]leucine into the protein of RN-P was exhibited by addition of 1 μM of ATP, 10 μM of phosphocreatine, more than 0.1 mg/ml of ATP-creatine transphosphorylase, more than 0.1 μM of GTP, and 30-120 μM of KCl in the presence of 5 μM of $MgCl_2$ and at pH 7.8–8.0.

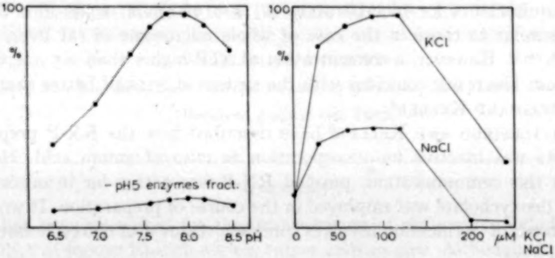

Fig. 6. The effect of pH and salt concentration on the incorporation of L-[^{14}C]leucine. The incubation medium was the complete system described in Table II, minus Tris buffer of pH 7.8 or KCl. To change the value of pH, Tris buffer of different pH was used. Incubation time, and longitudinal scale of figure were the same as in Fig. 5.

DISCUSSION

Many authors[2,7,10] have used Na-deoxycholate for the purpose of preparing RN-P from the microsomal fraction of animal tissues, but further purification of RN-P from the deoxycholate-insoluble fraction was difficult because such crude RN-P fraction was extremely unstable and readily caused flocculent precipitation owing to the dissociation of the complex. At the same time, a metalloprotein, the sedimentation coefficient of which was approximately 60–70 S contaminated this RN-P fraction[10,15]. Hence, PETERMANN AND HAMILTON[10] have used a stabilizing agent from cell dialysate for the purification.

In contrast with such instability of the crude RN-P fraction, purified RN-P preparation was surprisingly stable and the sedimentation pattern of the preparation stored for two months after freeze-drying was quite similar to that of the original preparation. Furthermore, freeze-dried RN-P exhibited full amino acid incorporation activity. That purified RN-P is more stable than crude preparation is considered to be due to the complete removal of contaminating enzymes, such as RNAase, from the soluble fraction of the cytoplasm.

The sedimentation analysis of the preparation revealed the main peak at 79 S and three additional peaks at 39 S, 58 S, and 116 S. Such components appear to correspond with A, B, C, and E components of PETERMANN AND HAMILTON's classification[10]. However, the assumption that three peaks at 58 S, 79 S, and 116 S are aggregates of the 39-S unit and that the divalent ions, especially Mg, are involved in the linkage of the RN-P units is essentially in agreement with the results obtained with RN-P of pea seedlings by Ts'o *et al.*[17] and of yeast by CHAO AND SCHACHMAN[1]. When the approximate molecular weight of four units are estimated assuming them to be spherical particles, the ratios of molecular weights will become roughly 1:2:3:6, for 39-S, 58-S, 79-S, and 116-S components. Therefore, it will be presumed that the 116-S component possibly dissociates to two 79-S units (1/2 of 116-S unit), the 79-S unit to a 39-S and a 58-S units (1/3 and 2/3 of 79-S unit) and the 58-S unit to two 39-S units (1/2 of 58-S unit). Reports of relatively low sedimentation coefficients on the microsomal RN-P[7,8,15] are considered to be due to the fact that the preparation has been performed in a Mg-free medium.

The requirements for incorporation *in vitro* of amino acids into the purified RN-P are similar to those in the case of whole microsome of rat liver, as already reported[8,11,14,19]. However, a concentration of ATP higher than 2.5 μM inhibits the incorporation. The result coincides with the system of SACHS[11] better than with that of LITTLEFIELD AND KELLER[8].

LITTLEFIELD AND KELLER[8] have described how the RN-P prepared using deoxycholate was inactive for incorporation *in vitro* of amino acid. However, as reported in this communication, purified RN-P was active for incorporation even though the deoxycholate was employed in the course of preparation. It would appear that the process of purification involves complete removal of deoxycholate used.

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INCORPORATION OF IODODEOXYURIDINE INTO THE DEOXYRIBONUCLEIC ACID OF MOUSE EHRLICH-ASCITES-TUMOR CELLS *IN VIVO*

WILLIAM H. PRUSOFF

Department of Pharmacology, Yale University School of Medicine, New Haven, Conn. (U.S.A.)

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SUMMARY

Evidence has been presented for the incorporation of radioactive iododeoxyuridine into the DNA of mouse Ehrlich ascites tumor cells *in vivo*. Although thymidine was utilized preferentially (to a 40-fold greater extent than iododeoxyuridine) for the biosynthesis of DNA, an almost identical incorporation of iododeoxyuridine occurred when the total amount of [^{131}I]iododeoxyuridine injected was increased by 12-fold and the frequency of administration of the analogue was doubled.

INTRODUCTION

Incorporation has been demonstrated of 5-chloro-, 5-bromo- and 5-iodouracil into the deoxyribonucleic acid (DNA) of bacteria[1-6], and of 5-fluorouracil into mammalian ribonucleic acid (RNA)[7,8]. 5-iododeoxyuridine was prepared[9] and preliminary reports of its utilization for the biosynthesis of mammalian DNA *in vitro*[10-12] and *in vivo*[10,13] have appeared. In this report, evidence will be presented for the incorporation of IUdR into the DNA of mouse Ehrlich ascites tumor cells *in vivo*.

METHODS AND RESULTS

[^{131}I]iododeoxyuridine was prepared by the method described previously for the preparation of [5-^{131}I]iodouridine[14], with the substitution of deoxyuridine for uridine.

Incubation of mouse Ehrlich ascites cells with [^{131}I]IUdR, and subsequent fractionation by the SCHNEIDER modification[15] of the SCHMIDT-THANNHAUSER procedure[16], resulted in the appearance of the major portions of the radioactivity in the DNA-fraction of the cell (Table I). However, an appreciable concentration was observed also in the protein fraction, a finding which indicated that significant degradation of IUdR probably had occurred; that this is indeed the case was demonstrated by the extensive renal excretion of the ^{131}I as free iodide[17]. Presumably, the latter is utilized by the cell for the iodination of appropriate aromatic amino acids.

In order to establish that the radioactivity present in the DNA fraction was in the form of IUdR the following investigations were performed. Thirty mice bearing

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Abbreviations: IUdR, iododeoxyuridine; DNA, deoxyribonucleic acid.

TABLE I

DISTRIBUTION OF RADIOACTIVE IODINE INTO VARIOUS FRACTIONS DERIVED FROM MOUSE EHRLICH ASCITES TUMOR CELLS INCUBATED WITH [5-^{131}I]IODODEOXYURIDINE *in vitro* AND *in vivo*[*]

The reaction mixture consisted of packed cells (0.25 ml), pig serum (0.1 ml), [5-^{131}I)iododeoxyuridine (1 μmole, 1 μC), and TOTTER's modification of CHAMBERS' solution[29] to 2.6 ml. The incubation was conducted in triplicate in 20-ml beakers in a Dubnoff metabolic shaker at 37° (air); agitation at 90 cyc./min for 4 h.

Fraction	*Radioactivity per g of cells*	
	In vitro counts/min	*In vivo counts/min*
Lipid	None	None
RNA	72	220
DNA	560	2,800
Protein	270	2,000

[*] Details are described in the text.

4-day-old Ehrlich ascites tumors were injected twice with [^{131}I]IUdR (5 μmoles; 5 μC) at 24-h intervals and sacrificed 24 h following the latter injection. The ascites cells were collected by centrifugation, and highly polymerized DNA (156 mg) was isolated by the procedure described by CHARGAFF[18]. The ratio of the u.v.-absorption of this preparation of DNA at 260 to 280 mμ was 1.64, and upon enzymic hydrolysis to the nucleotide level the ratio was increased to 1.82. The DNA was hydrolyzed to oligonucleotides by incubation with deoxyribonuclease at pH 6.5, and then to the nucleotide stage by hydrolysis with snake venom phosphodiesterase at pH 8.5, according to the method described by SINSHEIMER AND KOERNER[19]. The nucleotides were separated by ion exchange chromatography, using Dowex-1-formate (4 ×, 30 × 2.5 cm) in the manner described by CANELLAKIS AND MANTSAVINOS[20]. The eluates were examined for u.v.-absorption at 260 mμ in a Beckman spectrophotometer, and for γ-radiation in a well-type scintillation counter. Essentially all radioactivity was found in a single fraction located between adenylic and guanylic acids (Fig. 1-A). The higher affinity for Dowex-1 of "5-iododeoxyuridylic acid," as compared to thymidylic acid, was not unexpected because the replacement of the methyl group of thymidine by an iodine atom resulted in a lowering of the pK_a from 9.8 to 8.2, as determined by electrometric titration.

The formate was removed from radioactive "iododeoxyuridylic acid" by adsorption of the nucleotide on a charcoal column (5 × 1 cm) followed by elution with alcoholic ammonia (2.5 % NH_3, 50 % EtOH). The eluate was concentrated *in vacuo* to a small volume, diluted with water, and again reduced in volume. The solution of "iododeoxyuridylic acid" was adjusted to pH 5.2 and incubated with prostatic phosphatase in order to form the corresponding nucleoside. Identification of the product of the enzymic reaction as [^{131}I]IUdR was made on the basis of ion exchange and paper chromatography, in comparison with a standard preparation of IUdR. The radioactive areas coincided with the u.v.-absorbing areas of IUdR upon paper chromatography using the isopropyl alcohol–HCl system[21], the butanol–formic acid system[22], and the ethyl acetate–phosphate buffer system[23] with R_F values of 0.76, 0.55 and 0.44, respectively. Non-radioactive IUdR was added to an aliquot of the tentatively identified [^{131}I]IUdR, the pH was adjusted to 10.5 and the material was adsorbed on a Dowex-1-formate column (30 × 2.5 cm). Upon elution with 0.1 *N* formic acid the pattern of removal of the radioactive material coincided with that of iododeoxyuridine. When $[^{131}\text{I}]$IUdR was mixed with deoxycytidylic acid and deoxyadenylic acid, adsorbed on a Dowex-1-formate column (30 × 2.5 cm), and eluted by the procedure described by CANELLAKIS AND MANTSAVINOS[20], IUdR and deoxycytidylic acid were eluted essentially simultaneously (Fig. 1-B).

Additional evidence that the radioactivity in DNA was present originally in a polymerized form is shown in Table II. The radioactivity was converted into a dialyzable form only after digestion with deoxyribonuclease[19].

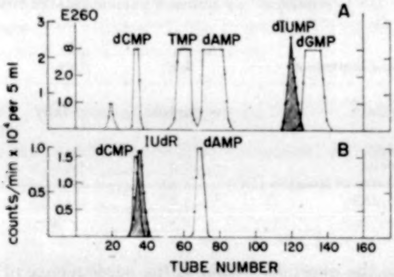

Fig. 1. A. Elution pattern of deoxyribonucleotide components of DNA of Ehrlich ascites tumor cells from mice treated with $[5\text{-}^{131}\text{I}]$iododeoxyuridine. B. Elution pattern of mixture of $[5\text{-}^{131}\text{I}]$-iododeoxyuridine, deoxycytidylic acid and deoxyadenylic acid. Peaks indicate magnitude of absorption at 260 mμ, while cross-hatched areas indicate occurrence of radioactive zones (see text for details).

TABLE II

EVIDENCE FOR THE INCORPORATION OF $[5\text{-}^{131}\text{I}]$IODODEOXYURIDINE INTO POLYMERIZED DNA

Dialyzed twice against 40 volumes of 3% NaCl solution for 24 h at 5°.

Substance dialyzed	*Per cent of radioactivity in dialysate*
Polymerized $[^{131}\text{I}]$DNA	1.0
Deoxyribonuclease digest of $[^{131}\text{I}]$DNA	95.0

The amount of $[^{131}\text{I}]$IUdR utilized for the biosynthesis of the above preparation of DNA was equivalent to a replacement of 0.56% of the thymidine-complement of the DNA. However, the $[^{131}\text{I}]$IUdR was administered under conditions which required that a major portion of the DNA isolated had been synthesized prior to incorporation of the analogue. Therefore, it was of importance to determine whether the amount of the analogue utilized for DNA-synthesis could be increased and to compare its uptake with that of thymidine. Two groups of mice were given equimolar amounts (2 μmoles) of either $[^{14}\text{C}]$thymidine or $[^{131}\text{I}]$IUdR during a period of 4 days, and, as shown in Table III, there was a 40-fold greater utilization of thymidine, than of its analogue, for the biosynthesis of DNA. However, by a 12-fold increase in the amount of $[^{131}\text{I}]$IUdR administered, and by dosage at 12-h rather than 24-h intervals, the extent of incorporation into DNA approached that of thymidine

(Table III). Thus, in the latter experiment the amount of incorporation of IUdR into DNA, although equivalent to only a 4.1 % replacement of the thymidine-complement of the DNA, it was equivalent to 90 % of the uptake of thymidine observed in the former experiment. Hence, in the calculation of the incorporation of an analogue into the cell in this type of experiment, the absolute value has but little significance, as compared to the relative extent of the incorporation of the normal metabolite and the corresponding analogue.

TABLE III

COMPARISON OF THE UTILIZATION OF RADIOACTIVE THYMIDINE AND 5-IODODEOXYURIDINE FOR THE BIOSYNTHESIS OF DNA-"THYMINE" OF MOUSE EHRLICH ASCITES TUMOR CELLS *in vivo*

	Exp. 1		*Exp. 2*
Compound administered	*TdR*	*IUdR*	*IUdR*
Regimen—4 days	0.5 μmole *i.p.* once daily		3.0 μmoles *i.p.* twice daily
Number of mice	10	10	9
Wet weight cells	12.8	15.4	12.5
260/280 u.v.-ratio of isolated DNA	1.55	1.6	1.5
"Thymine" uptake	4.6 %	0.12 %	4.1 %

Of importance is the question "What is the consequence of the presence of this unnatural pyrimidine in the DNA-polymer on its biological acitivity". MANTSAVINOS[24], in a recent investigation in this department of the primer activity of DNA derived from various sources, studied a preparation of DNA which contained IUdR derived from Ehrlich ascites tumor cells and found no evidence for decreased primer activity. Since DNA represents a heterogeneous population one does not know whether the activity observed was due to the iodine-containing DNA, the iodine-free DNA, or both. Even if the iodinated DNA were inhibitory it may not have been present at a concentration sufficiently high to manifest itself.

Although both 5-iodouracil and IUdR have been shown to be inhibitors of cellular growth[4,9,11,12,25,26], iodouracil, in the presence of suboptimal concentrations of thymidine, will increase by 4-fold the amount of growth of a mutant strain of *Streptococcus faecalis*[27]. Iodouracil can be incorporated into the DNA of bacteria and hence it may be assumed that iodouracil can substitute for thymine in at least some of its metabolic activities.

DISCUSSION

In agreement with studies performed with bacteria, it appears that analogues of certain pyrimidines also may be utilized by some mammalian systems. Whether this incorporation, with the resultant formation of a fraudulent DNA, is the explanation for the anti-tumor activity of IUdR[26], is not yet clear. Studies with another pyrimidine analogue, 6-azathymine, indicated a lack of correlation between incorporation into the DNA of *S. faecalis* and cellular death[28]. Although iodouracil can partially replace thymidine in support of the growth of a mutant form of *S. faecalis*[27], IUdR is a competitive antagonist of thymidine in support of the growth of *S. faecalis*[27], and of the utilization of thymidine for the biosynthesis of the DNA of Ehrlich ascites tumor cells[9]. Also, thymidine prevents the toxicity of IUdR in mice[17]. Thus, a relationship between thymidine and IUdR (or their phosphorylated derivatives) has been observed, but precisely which of the many metabolic reactions, in which these compounds may be involved, serves as the foundation for the observed biological result, remains to be elucidated.

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MECHANISM OF DEPLETION OF MITOCHONDRIAL PYRIDINE NUCLEOTIDES*

BERNARD T. KAUFMAN AND NATHAN O. KAPLAN

Graduate Department of Biochemistry, Brandeis University, Waltham, Mass. (U.S.A.)

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SUMMARY

Studies have been carried out on the mechanism of inactivation of pyridine coenzymes in rat-liver mitochondria in the presence of inorganic phosphate and other agents. The inactivation has been found to occur through three stages: (a) the oxidation of the reduced coenzymes, primarily reduced TPN, (b) the leakage of the oxidized pyridine coenzymes from the mitochondria, and (c) the cleavage of the oxidized forms by an extra-mitochondrial DPNase.

A correlation has been established between the oxidation of the reduced coenzymes and the swelling of the mitochondria. Substances which inhibit the swelling also suppress the oxidation. The destruction of the pyridine nucleotides is a secondary effect and is not related to the swelling process. Nicotinamide which inhibits the destruction of the coenzymes has no effect on the oxidation, release of the pyridine nucleotides or swelling.

Succinate has been found to inhibit the release of the pyridine coenzymes as well as the swelling of the mitochondria. Malonate abolishes this effect of succinate. In addition to succinate, isocitrate and glutamate as well as cyanide and amytal are capable of suppressing the loss of the pyridine nucleotides from the mitochondria.

Reduced coenzymes have not been observed to be lost directly from the mitochondria. Oxidation of the reduced forms appears to be essential before their release and destruction.

INTRODUCTION

The inactivation of pyridine nucleotide-dependent oxidations of rat-liver mitochondria by the aerobic incubation of the particles with phosphate, pyrophosphate, arsenate, arsenite, Zn^{++}, Ca^{++} or surface active agents, and the restoration of activity by DPN, led HUNTER *et al.*[1,2] to the conclusion that a loss or destruction of pyridine nucleotides had occurred. The effects of certain of these agents could be prevented by the presence of EDTA, Mn^{++}, Mg^{++}, or by the exclusion of oxygen[1]. Furthermore,

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The abbreviations used are DPN and TPN for diphosphopyridine nucleotide and triphosphopyridine nucleotide, respectively; DPNase, diphosphopyridine nucleotidase; EDTA, ethylenediaminetetraacetate; TRIS, tris(hydroxymethyl)aminomethane.

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changes in the structural integrity of the mitochondria as evidenced by swelling appeared to be related to the loss of oxidative reactivity. KAPLAN *et al.*[3] demonstrated that "depleted" mitochondria do not contain either the oxidized or reduced forms of DPN but may contain traces of TPN. A similar loss of pyridine nucleotides was shown to occur in beef-heart particles[4]. EMMELOT AND BOS[5] also concluded that the inhibition of glutamate oxidation in mouse-liver mitochondria by thyroxine is mediated by a release of DPN from the mitochondrial dehydrogenase. In this case, EDTA, Mn^{++} or Mg^{++} also counteracted the effects of thyroxine. The destruction of the activity of mitochondria by treatment with Ca^{++} was explained by ERNSTER[6], in a similar manner, to be due to the loss or destruction of the bound pyridine nucleotides.

The similar characteristics of the various inactivation phenomena suggest that a common mechanism of action may be involved which in turn may be related to the nature of the intra-mitochondrial binding of the pyridine nucleotides. Therefore, in the present study the mitochondrial pyridine nucleotides and the mitochondria have been investigated under conditions leading to the loss of the codehydrogenases. Studies on conditions which protect the bound pyridine nucleotides will also be reported.

METHODS

Rat liver, after being forced through a tissue press, was ground gently in an ice-cold Ten Broeck homogenizer with 10 volumes of 0.25 *M* sucrose. The homogenate was centrifuged at 600 × *g* for 10 min to remove the easily sedimentable materials. The homogenate supernatant fluid was then centrifuged at 6,000 × *g* for 10 min in order to isolate the mitochondria. The pellet was washed twice by recentrifugation of the resuspended material at approx. 24,000 × *g*. The final mitochondrial suspension was made in 0.25 *M* sucrose so that 1 ml represents 0.5 g of original liver. Pigeon-liver mitochondria were isolated in an identical manner. Special precautions were taken to maintain all manipulations at 0° prior to the incubations of the final suspension.

Mitochondria were incubated as follows. To 2.0 ml of the above suspension in test tubes was added 0.5 ml of 0.25 *M* sucrose containing 0.1 *M* potassium phosphate (pH 7.5). The tubes were incubated for the times indicated with constant shaking at 30°. The tubes were then returned to ice and immediately assayed. Trichloroacetic acid was added to a final concentration of 5 % to the tubes to be analyzed for oxidized pyridine nucleotides. The contents of tubes to be assayed for reduced pyridine nucleotides were rapidly added to a glass hand homogenizer containing 2.5 ml of boiling 0.1 *M* Na_2CO_3 (pH 10), quickly mixed and allowed to remain in the boiling bath for 1 min before returning to ice and diluted to 5 ml. Leakage of pyridine nucleotides from the mitochondria was determined by immediately centrifuging the chilled incubation mixture at 24,000 × *g* for 5 min and treating the supernatant fluid with either trichloroacetic acid or Na_2CO_3 as previously described.

Where indicated, the phosphate in the 0.5 ml of 0.25 *M* sucrose was replaced by thyroxine, Zn^{++} or Ca^{++} in the following concentrations: $2.5 \cdot 10^{-4}$ *M*, $3 \cdot 10^{-4}$ *M* and $3 \cdot 10^{-3}$ *M* respectively. When used, additions to the phosphate-containing incubation media were made at the following final concentrations: EDTA, $2 \cdot 10^{-3}$ *M*; nicotinamide, 0.05 *M* and $MgCl_2$, $3 \cdot 10^{-3}$ *M*. When added, 0.05 ml of DPNase, prepared from zinc-deficient *Neurospora crassa* by the method of KAPLAN *et al.*[7], was used. Other additions are noted in the appropriate tables.

Respiration and phosphate uptake were determined as previously described[3]. Swelling was measured by resuspending 0.05 ml of the incubation mixture, at the times indicated, in 2.95 ml of 0.25 *M* sucrose containing 0.05 *M* EDTA in a 1-cm cuvette and determining the O.D. at 520 mμ. Pyridine nucleotides were determined in the acid or alkaline extracts of the total incubation mixture or resulting mitochondrial supernatants by the specific enzymic-fluorometric procedures described by JACOBSON AND ASTRACHAN[8].

RESULTS

In order to obtain definitive information on the pyridine nucleotides during the depletion and inactivation of mitochondria, a study was made of the changes of the total endogenous oxidized pyridine nucleotides of mitochondria incubated in the presence and absence of inorganic phosphate. Fig. 1a illustrates the behavior of the pyridine nucleotides of freshly prepared rat-liver mitochondria suspended in 0.25 *M* sucrose containing 0.02 *M* phosphate and incubated aerobically at 30° with constant shaking. The marked rise in the fluorescence of the total incubation mixture during the first 10 min of the reaction indicates a rapid increase in the level of the oxidized pyridine nucleotides. The decline in the fluorescence represents the destruction of the codehydrogenases and corresponds to the depletion of coenzymes from the mitochondria. At the same time the mitochondria begin to swell as shown by a decrease in the turbidity of the suspension (Fig. 1b). In general, a small but definite lag is observed between the maximal appearance of the oxidized pyridine nucleotides and swelling of the mitochondria. These changes do not occur at 0°. Furthermore, freshly prepared mitochondria are found to be resistant to short-term preincubations in the absence of inorganic phosphate. In general, no significant changes are observed in

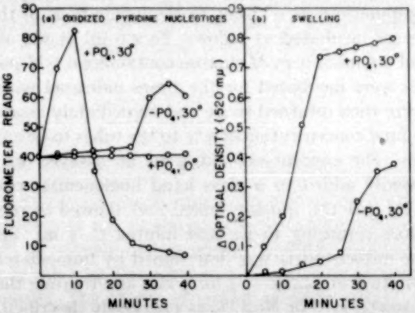

Fig. 1. Effect of aerobic incubation of rat-liver mitochondria in the presence and absence of 0.02 *M* inorganic phosphate on the (a) total oxidized pyridine nucleotides and (b) swelling of the mitochondria. Values for the oxidized pyridine nucleotides are expressed as the relative fluorescence of an acid extract of the total incubation mixture treated with methyl ethyl ketone as described in methods. Swelling of the mitochondria is expressed as the decrease in absorbancy of a 0.05-ml aliquot of the mitochondrial incubation mixture resuspended in 2.95 ml of isotonic sucrose containing EDTA.

the mitochondrial pyridine nucleotides even after 15 min of incubation. Similarly, the structural alteration of the mitochondria parallels the behavior of the coenzymes. However, prolonged incubation in isotonic sucrose alone is characterized by an increase in oxidized forms and spontaneous swelling. If the incubation is carried out for sufficiently long periods of time, a decline in the methyl ethyl ketone fluorescence eventually occurs, indicating that the pyridine nucleotides are lost. The pyridine nucleotide loss and swelling obtained in the absence of phosphate are somewhat variable with respect to the incubation time required to attain the maximum effect. Furthermore, it should be noted that the maximum swelling and fluorescence attained in the absence of phosphate is significantly less than that observed in the presence of this reagent. In addition, mitochondria which have been stored for 24 h at 0° show a marked increase in the rate of depletion or destruction of the pyridine nucleotides in the absence of phosphate. Such aged mitochondria also exhibit a significantly higher initial level of oxidized coenzymes, indicating that the above changes may have occurred to a certain extent during storage.

Oxidation and destruction of mitochondrial pyridine nucleotides

The data presented in Fig. 2 demonstrate that the variation in the total oxidized pyridine coenzymes is a result of the increase and subsequent disappearance of both DPN and TPN. This increase in oxidized coenzymes has been previously noted by JACOBSON AND KAPLAN[9], and is due to the oxidation of the intra-mitochondrial reduced pyridine nucleotides as illustrated in Fig. 2. The total increase is due chiefly to the conversion of TPNH to TPN, which is consistent with the observation that TPNH is the major pyridine nucleotide of carefully prepared rat-liver mitochondria[9]. Although it is evident that these mitochondria are rapidly depleted of the pyridine nucleotides under these conditions, traces of TPN persist during a short term incubation period because of the markedly higher initial content of this coenzyme.

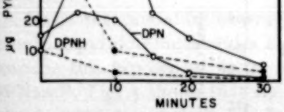

Fig. 2. Changes in the oxidized and reduced pyridine nucleotides during the aerobic incubation of rat-liver mitochondria in the presence of 0.02 M phosphate. Individual oxidized and reduced coenzymes were assayed in acid and alkaline extracts, respectively, of the total incubation mixture at the indicated times by the specific, enzymic fluorometric procedure as described in methods.

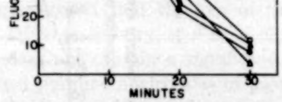

Fig. 3. Effects of aerobic incubation (30°) of rat-liver mitochondria in the presence of thyroxine, $5 \cdot 10^{-5}$ M; phosphate, 0.02 M; Ca^{++}, $6 \cdot 10^{-4}$ M; or Zn^{++}, $6 \cdot 10^{-5}$ M on the total oxidized pyridine nucleotides. Values represent the codehydrogenases in the total mitochondrial incubation mixture.

Other agents reported to affect mitochondrial oxidations were examined for their ability to deplete the mitochondria of pyridine nucleotides. Fig. 3 shows that incubation of mitochondria in the presence of thyroxine, Ca^{++} or Zn^{++} affects the pyridine nucleotides in a manner essentially similar to that occurring in the presence of phosphate. The primary effect of these substances, regardless of their chemical nature, is to cause a rapid oxidation of the mitochondrial pyridine nucleotides followed by the characteristic disappearance of the coenzymes. Calcium appears to cause more extensive damage to the system since swelling takes place immediately upon the addition of the agent followed by extensive aggregation and precipitation of the mitochondria.

Inhibition of mitochondrial depletion

The depletion of mitochondrial pyridine coenzymes induced as described above can be prevented by the exclusion of oxygen[2] or the addition of EDTA, Mg^{++} or Mn^{++} to the incubation medium[1]. If the primary step in the depletion process is the oxidation of the pyridine nucleotides, the inhibition by anaerobic conditions can be understood on this basis. Fig. 4 shows that EDTA and Mg^{++} also prevent the phosphate induced oxidation of the pyridine nucleotides as well as the swelling of the particles. Consequently, the depletion of the mitochondrial pyridine nucleotides does not occur. EDTA is only partially effective in this respect after the mitochondria have been stored overnight at 0°. In confirmation of the report of SEVERIN[10], anserine and carnosine were also observed to protect the mitochondrial pyridine nucleotides from the effects of incubation with phosphate. However, glycylglycine is equally effective. TRIS has a slight protective effect. As in the case of EDTA, these compounds probably function by virtue of their metal binding properties.

Fig. 4 also illustrates the protective action of nicotinamide. Although the destruction of the pyridine nucleotides is essentially completely inhibited, the rapid oxidation of the reduced pyridine coenzymes and the mitochondrial swelling are not suppressed. These observations with rat-liver mitochondria tally closely with those of LESTER AND HATEFI with beef-heart particles[4]; the protective effect, however, of

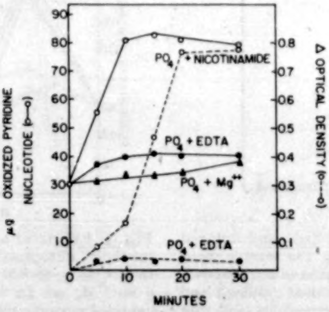

Fig. 4. Effect of nicotinamide (○), 0.05 M; EDTA (●), $2 \cdot 10^{-3}$ M; and Mg^{++} (▲), $3 \cdot 10^{-3}$ M, on the oxidation of the pyridine nucleotides (solid line) and swelling (broken line) of rat-liver mitochondria incubated in the presence of 0.02 M phosphate. O.D. changes measured at 520 mµ (see Fig. 1).

EDTA appears to be different in the rat-liver particles. We have observed that EDTA consistently prevents the oxidation of the reduced pyridine nucleotides in the rat-liver preparations.

Despite the presence of what appears to be the normal complement of total pyridine nucleotides in the mitochondrial suspension after preincubation in the presence of inorganic phosphate and nicotinamide, the mitochondria are essentially inactive with respect to oxidative phosphorylation unless further supplemented with a large excess of DPN (Table I). This loss of activity is not due to the presence of nicotinamide since its addition to the Warburg assay medium does not affect significantly the activity of normal mitochondria. Furthermore, it does not appear that the pyridine nucleotides have been altered in any way by this procedure since the high level of fluorescence is rapidly destroyed by the DPNase from *Neurospora crassa*. It would appear that this uncontrolled oxidation of the pyridine coenzymes results in a release of the bound pyridine nucleotides from the mitochondrial dehydrogenases and a higher than normal level of DPN is necessary to reestablish the oxidative reactions of the mitochondria.

TABLE I

EFFECT OF NICOTINAMIDE ON DEPLETION AND OXIDATIVE PHOSPHORYLATION OF RAT-LIVER MITOCHONDRIA

Preincubation conditions	*Addition to flask[*] µmoles*	*Oxygen uptake µatoms*	*Phosphorus uptake µmoles*	*P/O*
None		8.7	25.4	2.9
Phosphate, 0.02 *M*		0	0	—
Phosphate, 0.02 *M* + nicotinamide, 0.05 *M*		4.2	3.7	0.9
Phosphate, 0.02 *M* + nicotinamide, 0.05 *M*	DPN (1.5)	16.5	31.0	1.9
None	Nicotinamide (10)	6.8	20.0	2.9

[*] Each Warburg flask contains the following substances to give the indicated final concentration in 3.0 ml total mixture: 0.0017 *M* ATP, 0.02 *M* NaF, 0.005 *M* $MgCl_2$, 0.01 *M* KCl, 0.005 *M* gly·gly (pH 7.4), 0.001 *M* EDTA (pH 7.5), 0.013 *M* potassium phosphate (pH 7.5), 0.007% cytochrome *c* and 0.01 *M* sodium glutamate. The reaction is started by the addition of glucose, 0.02 *M* final concentration, and 360 Cori units of yeast hexokinase. Other additions are indicated in table.

Depletion of pigeon-liver mitochondria

Since the preceding observations strongly suggest that the loss of the mitochondrial pyridine nucleotides is a result of DPNase activity, it was of interest to examine this process in pigeon liver, which does not contain a significant level of DPNase[11]. Fig. 5 shows that the total oxidized pyridine nucleotides of pigeon-liver mitochondria do not change during incubation in the presence or absence of inorganic phosphate. The lack of any significant increase in the oxidized forms of the coenzymes as compared with rat-liver mitochondria confirms the observation that the pyridine nucleotides of pigeon-liver mitochondria surviving isolation are almost totally in the oxidized form[9]. However, if *Neurospora* DPNase is added to the incubation medium, the pyridine nucleotides are rapidly destroyed. In the presence of EDTA the destruction of the pyridine nucleotides by the added DPNase is significantly inhibited.

Since EDTA does not affect the activity of *Neurospora* DPNase, it must function in this system by suppressing the leakage of the coenzymes from the mitochondria to the medium where they can be attacked by the added DPNase. The leakage of the pyridine nucleotides from the mitochondria must be the limiting step in the depletion process since the addition of DPNase to the rat-liver mitochondrial incubation medium in the presence or absence of inorganic phosphate has no significant effect on the rate of depletion.

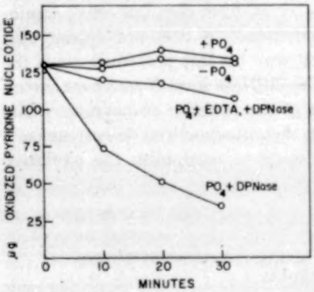

Fig. 5. Effect of EDTA and/or DPNase on the depletion of the pyridine nucleotides of pigeon-liver mitochondria incubated in the presence of 0.02 *M* phosphate. Values represent the oxidized pyridine coenzymes in the total mitochondrial incubation mixture.

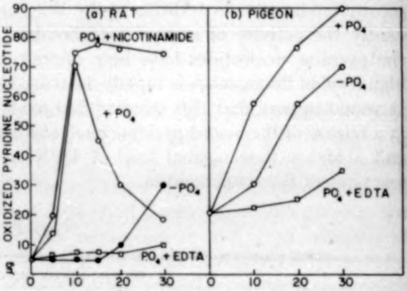

Fig. 6. Release of oxidized pyridine nucleotides into the medium during the aerobic incubation (30°) of (a) rat liver and (b) pigeon-liver mitochondria under conditions noted in the figure and described in METHODS. Values represent total pyridine nucleotides in supernatant fluid after centrifugation of the standard mitochondrial incubation mixture at the specified times.

Leakage of pyridine nucleotides from mitochondria

That the pyridine nucleotides do leak out of the mitochondria during the incubation is shown in Figs. 6a and 6b which illustrate the rapid appearance of the pyridine coenzymes from rat- and pigeon-liver mitochondria in the incubation medium. In the case of rat-liver mitochondria, the leakage parallels almost exactly the rate of oxidation of the coenzymes. However, consideration of Fig. 1b appears to indicate that the pyridine nucleotides have completely leaked from the mitochondria before the maximum rate of swelling begins. As would be expected, EDTA prevents the release of the pyridine nucleotides. The effectiveness of EDTA in suppressing the leakage is greatly diminished after the mitochondria have been stored overnight at 0°. Incubation in the absence of phosphate does not result in a significant release of the pyridine nucleotides until the oxidation of the coenzymes and the swelling of the mitochondria begin. Since no reduced pyridine nucleotides can be detected in the medium, the oxidation of the coenzymes seems to be a necessary prerequisite for leakage to occur. Despite the lack of any significant variation in the total oxidized pyridine nucleotides of pigeon-liver mitochondria when incubated in the presence of phosphate (Fig. 5), Fig. 6b indicates that phosphate does enhance the rate of leakage from the mitochondria. However, it should be noted that a significant release of pyridine coenzymes takes place in the absence of phosphate.

Site of pyridine nucleotide destruction

The release of almost, if not all, of the pyridine nucleotides from the mitochondria within the first 10 min of the incubation period precludes the destruction of the coenzymes within the mitochondria. This is consistent with the observation that rat-liver mitochondria do not contain significant DPNase activity[11]. As the bulk of rat liver DPNase is associated with the microsomal fraction, attempts were made to remove the DPNase by washing the mitochondria by repeated centrifugation at 6,000 × *g*. Table II shows that the rate of destruction of the pyridine nucleotides is significantly decreased by washing the mitochondria; however, a limiting value is approached which suggests that a certain fraction of the destructive activity is apparently associated with the mitochondria or the mitochondrial surface. Activation of a latent DPNase by the incubation procedure could not be demonstrated.

TABLE II

EFFECT OF WASHING ON THE RATE OF DEPLETION OF RAT-LIVER MITOCHONDRIA INCUBATED IN THE PRESENCE OF 0.02 *M* PHOSPHATE

Number of washings 6,000 × g	*Disappearance of pyridine nucleotides µg/min**
1	0.90
2	0.63
3	0.50
4	0.47

* Values represent rate of disappearance of total oxidized pyridine nucleotides from standard incubation mixture.

Inhibition of release of mitochondrial-bound pyridine nucleotides

Since the above observations suggest that the reduced state of the pyridine nucleotides, particularly reduced TPN, is essential for the maintenance of intramitochondrial binding, substances which directly affect the oxidation or reduction of the respiratory carriers should alter the effects of phosphate. The addition of substrates to rat-liver mitochondria incubated in the presence of phosphate and nicotinamide confirms, in terms of a suppression of pyridine nucleotide leakage, the well known stabilization of mitochondrial activity by substrates of the Krebs cycle. Table III shows that succinate is the most effective in this respect while β-hydroxybutyrate is the least effective. Furthermore, isocitrate, a TPN-linked substrate, is consistently observed to exhibit a greater protection than glutamate.

Although it has been reported that cyanide does not protect against the loss of DPN[3], it was found that somewhat higher concentrations of cyanide (0.01 *M*) do suppress the release of the pyridine nucleotides (Table III). Amytal, which inhibits oxidations linked with the pyridine coenzymes[12], effectively prevents the release at a concentration of 0.004 *M*. On the other hand, in the presence of β-hydroxybutyrate which is relatively ineffective alone, lower concentrations of both cyanide and amytal inhibit the release of the coenzymes.

It can be seen from Table IV that the inhibitory effects of succinate are dependent on its metabolic functions since malonate reverses this protection. Malonate alone has no significant effect on the phosphate induced release of the pyridine nucleotides.

In marked contrast to the observations of previous investigators[13–15], it can be seen that under these conditions of mitochondrial concentration and incubation, succinate also inhibits the swelling of the particles and this protection is in turn reversed by malonate.

TABLE III

SUBSTANCES AFFECTING THE RELEASE OF PYRIDINE NUCLEOTIDES FROM RAT-LIVER MITOCHONDRIA INCUBATED IN THE PRESENCE OF PHOSPHATE AND NICOTINAMIDE

Experiment	*Addition to incubation medium*	*Concn. (mM)*	*µg pyridine nucleotides* released in*	
			10 min	*20 min*
1	None	—	85	83
	Succinate	20	10	28
	Isocitrate	20	22	47
	Glutamate	20	42	73
	β-hydroxybutyrate	20	62	79
2	None	—	89	80
	Cyanide	4.6	21	75
	Cyanide	10	11	15
	Amytal	2	43	78
	Amytal	4	6	21
3	None	—	79	78
	β-hydroxybutyrate	20	10	25
	plus cyanide	2		
	β-hydroxybutyrate	20	12	20
	plus amytal	2		

* Values represent total oxidized pyridine nucleotides in supernatant fluid after centrifugation of the mitochondrial incubation mixture at times indicated. (Conditions as described in Fig. 6.)

TABLE IV

EFFECT OF SUCCINATE AND MALONATE ON LEAKAGE OF PYRIDINE NUCLEOTIDES AND SWELLING OF RAT-LIVER MITOCHONDRIA INCUBATED IN THE PRESENCE OF PHOSPHATE AND NICOTINAMIDE

Addition to incubation medium	*µg pyridine nucleotides released**	*—Δ O.D. at 520 mµ**
None	64	0.710
Succinate**	14	0.095
Malonate	68	0.740
Succinate + Malonate	38	0.520

* 20-min incubation.
** Final substrate concentration, 20 m*M*.

DISCUSSION

The observations reported in this study suggest that the total oxidation of the intramitochondrial pyridine nucleotides, the release of the oxidized coenzymes from the mitochondria and swelling of the mitochondria observed during the incubation of the rat-liver particles in isotonic phosphate are all inter-related phenomena. However, the depletion or disappearance of the pyridine nucleotides is a secondary process which results from the leakage and subsequent destruction of the coenzymes by extra-mitochondrial DPNase. Thus, despite widely differing chemical properties of the depleting agents, it would appear that the rapid oxidation of the mitochondrial bound reduced pyridine nucleotides is a primary effect of these agents. A direct attack of these substances on the mitochondrial membrane cannot be ruled out; however, the pyridine nucleotides are observed to be released from the mitochondria prior to the occurrence of visible alteration, *i.e.* swelling. Furthermore, the inhibition by anaerobiosis of both swelling[16] and the inactivation of pyridine nucleotide dependent reactions[2] emphasizes the common link between the two processes. Although the swelling of mitochondria as described by LEHNINGER AND RAY[16] may be associated with members of the respiratory chain other than the pyridine nucleotides, the total oxidation of these codehydrogenases will in turn result in the alteration of the redox steady states of the other carriers.

Therefore, taking oxidation as the primary effect of the depleting agent, the protective effects of EDTA, Mg^{++} or Mn^{++} can be assumed to retard this process, whereas pyridine nucleotide-linked substrates play a key role by the maintenance of the coenzymes in the reduced state. The observation of CHANCE AND HOLLUNGER[17] that the mitochondrial pyridine coenzymes are rapidly reduced by succinate, is further evidence that the protective effect is mediated by the pyridine nucleotides. Although β-hydroxybutyrate alone is relatively ineffective, the marked increase in the suppression of leakage when cyanide or amytal and β-hydroxybutyrate are added to the depletion medium suggests that β-hydroxybutyrate is not capable of effectively reducing the pyridine nucleotides unless the respiratory chain is blocked. However, the relatively high concentrations of cyanide and amytal required to effectively inhibit the release of the pyridine nucleotides may be interpreted as indicating that the depleting agent, phosphate, partially uncouples the reduced pyridine nucleotides from the respiratory chain, allowing oxidation to proceed via a pathway which is not wholly sensitive to cyanide or amytal[18].

The stability of the reduced pyridine nucleotides of rat-liver mitochondria, as evidenced by the lack of oxidation during incubation in the absence of phosphate, indicates that the reduced forms surviving the isolation of the particles are tightly bound in an unreactive state. CHANCE[19] has shown that the rapid oxidation of such "controlled" or bound reduced pyridine nucleotides induced by Ca^{++} is inhibited by Mg^{++}. Since the preexisting oxidized pyridine nucleotides do not leak out of the mitochondria until the onset of the oxidation, it must be assumed that a breakdown in the mitochondrial structure occurs during this phase which results in a rapid release of the oxidized forms of the coenzymes and is followed by visible swelling. The significant leakage of pyridine nucleotides from pigeon-liver mitochondria incubated in the absence of phosphate implies a lack of coupling between reduced pyridine nucleotides and the mitochondria which is consistent with the observation that the reduced forms apparently do not survive the isolation procedure. However, a specific role of the mitochondrial membrane cannot be overlooked in view of the report that pyridine nucleotides are not released from beef-heart mitochondria despite extensive oxidation[4].

It is of interest that although nicotinamide inhibits the destruction of the oxidized coenzymes which have been lost from the mitochondria, the mitochondria are essentially inactive in terms of oxidative phosphorylation. We have found, as recently also reported by HUNTER *et al.*[20], that exceptionally high concentrations of pyridine nucleotides are required before the mitochondria can be replenished with their normal complement of pyridine coenzymes.

ACKNOWLEDGEMENTS

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Short Communications

Interference of biochemically important reducing agents with the diazotation of sulfanilamide

Reduced di- or triphosphopyridine nucleotides are the electron donors of nitrate and nitrite reductases. These enzymes are usually assayed by colorimetric determination of the amount of nitrite formed during or remaining after incubation[1]. The chemical basis for this colorimetric analysis is the diazotation of sulfanilamide and the coupling of the resulting diazonium salt to N-(1-naphthyl)-ethylenediamine dihydrochloride, yielding a red azo dye. The intensity of the color produced is proportional to the amount of nitrite present at the onset of the diazotation step[2].

EVANS AND NASON[3] observed that DPNH and TPNH interfere with this reaction and suggested deamination of the adenine moiety of the nucleotide as a possible cause of this effect. MEDINA AND NICHOLAS[4] subsequently proposed competition between DPNH or TPNH and NO_2^- "for the same site on the diazo compound" to explain this interference.

During our studies on a TPNH-dependent nitrate reductase we also noted this interference but have found no evidence for deamination of the purine moiety of the nucleotide. Table I shows that only the dihydropyridine structure of TPNH appears to be involved in the reaction which leads to interference with the nitrite test.

TABLE I

COLORIMETRIC DETERMINATION OF NITRITE[2]

To 2.8 ml water, containing 40 mμmoles NO_2^- and 160 mμmoles additive, 1 ml 1 % (w/v) sulfanilamide in 3 *N* HCl and 1 ml 0.02 % (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride were added. After 10 min the intensity of the resulting color was measured at 540 mμ in a Beckman DU spectrophotometer.

	Determination of nitrite in the presence of				
	Adenine	*Hypoxanthine*	*D-Ribose*	*TPN*	*TPNH*
mμ moles NO_2^- found:	39	40	39	38	28
	38	39	39	39	28
	39	38	41	40	28

Competition between NO_2^- and the reduced nucleotide for reaction with sulfanilamide was discounted on consideration of the large excess of the latter ($5.8 \cdot 10^{-5}$ moles) over TPNH ($1.6 \cdot 10^{-7}$ moles) and NO_2^- ($0–50 \cdot 10^{-9}$ moles) present in a typical assay system for nitrate reductase. Reaction with TPNH, therefore, would not appreciably diminish the amount of sulfanilamide available for reaction with NO_2^-.

We have found that interference with the colorimetric determination of nitrite

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Abbreviations: TPN, triphosphopyridine nucleotide; TPNH, reduced TPN; DPNH, reduced diphosphopyridine nucleotide; GSH, glutathione.

is not confined to reduced nucleotides alone but is also given by cysteine and reduced glutathione, and presumably by other biochemically important reducing agents. Table II shows that the α-amino groups of these compounds are not involved in the observed interference since the oxidized dimers tested were without effect.

TABLE II

COLORIMETRIC DETERMINATION OF NITRITE[2]

To 2.8 ml water, containing 40 mμ moles NO_2^- and 10 μmoles additive, 1 ml 1 % (w/v) sulfanilamide in 3 *N* HCl and 1 ml 0.02 % (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride were added. After 10 min the intensity of the resulting color was measured at 540 mμ in a Beckman DU spectrophotometer.

	Determination of nitrite in the presence of			
	Cystine	*Cysteine*	*Oxidized glutahione*	*Reduced glutathione*
mμ moles NO_2^- found:	39	29	40	11
	39	30	40	11

Since cysteine is used to stabilize nitrate reductase preparations we have determined quantitatively its interference with the nitrite test. Fig. 1 shows that the presence of cysteine in assay mixtures at levels of up to 1 m*M* does not interfere with the enzyme assay.

As with TPNH[4] the effect of these reducing agents on the nitrite test is negligible when they are added after the diazonium salt has been formed. Therefore the interference of TPNH, cysteine, and reduced glutathione must take place before or during

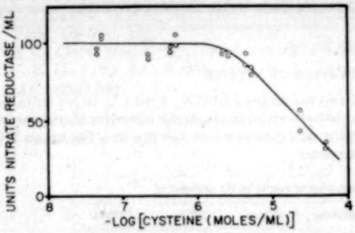


Fig. 1. Assay of nitrate reductase from *Neurospora crassa* 5297 A in the presence of cysteine. 1-ml assay mixture contained (in moles): FAD, 10^{-8}; KNO_3, $2 \cdot 10^{-5}$; TPNH, $1.6 \cdot 10^{-7}$; cysteine, and enzyme, in 0.1 *M* neutral phosphate buffer. Nitrite formed enzymically after 10 min of incubation is determined colorimetrically[2] in a Beckman DU spectrophotometer. Apparent units of nitrate reductase activity decrease with increasing cysteine concentration as a result of interference with nitrite analysis shown in Table II.

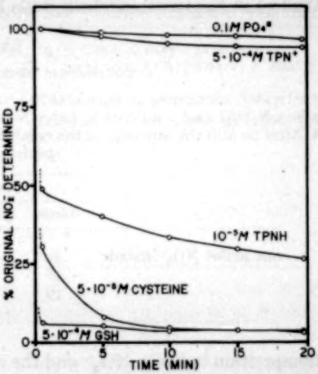
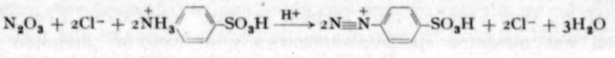


Fig. 2. Colorimetric determination of nitrite after exposure to reducing agents in 0.8 *N* HCl. To 2.0-ml portions of water containing 40 mμmoles NO_2^- and varying amounts of reducing agents were added 0.8 ml HCl to give a final normality of 0.8 *N*. At time intervals 1 ml 1 % (w/v) sulfanilamide in 3 *N* HCl and 1 ml 0.02 % (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride were added to each sample. After 10 min the intensity of the resulting color was measured at 540 mμ in a Beckman DU spectrophotometer. Additive concentrations shown refer to 2.8-ml reaction volume.

diazotation of sulfanilamide. We investigated the nature of this interference and found it to be the result of a competition between the reducing agent and sulfanilamide for reaction with nitrite which takes place in the acidic medium prevailing during diazotation. MEDINA AND NICHOLAS[4] found no evidence for a time-dependent interaction between TPNH and NO_2^-; however, the neutral pH of their experiment differs from the highly acid conditions of diazotation. We therefore studied the interaction between reducing agents and nitrite in the presence of 0.8 *N* HCl, the acid concentration during diazotation. We found that the period of contact was inversely related to the amount of nitrite which could subsequently be determined colorimetrically. This is shown in Fig. 2. From this evidence we conclude that under conditions of diazotation, where HCl and sulfanilamide are added simultaneously to a mixture of NO_2^- and reducing agent, a competitive situation is created in which nitrite may (a) react with sulfanilamide (equation 2a) or (b) be reduced by interaction with the reducing agent present (equation 2b). Rate and extent of the latter reaction appears to be a function of both the nature of the reducing agent and of its concentration.

$$2\,NO_2^- + 2H^+ \longrightarrow N_2O_3 + H_2O \qquad (1)$$



(2a)

$$N_2O_3 + \text{reducing agent} \xrightarrow{H^+} \text{oxidized reducing agent} + \text{reduced } N_2O_3 \qquad (2b)$$

In the case of reducing agents containing sulfhydryl groups, reaction (2b) may result in the formation of a thioester of nitrous acid[5] (equation 3) which may decompose to give a disulfide and nitric oxide (equation 4).

$$2RSH + N_2O_3 \longrightarrow 2RSNO + H_2O \qquad (3)$$

$$2RSNO \longrightarrow RSSR + 2NO \qquad (4)$$

With TPNH as the reducing agent reaction (2b) may formally result in hyponitrous acid according to

$$2TPNH + N_2O_3 + 2\,H^+ \longrightarrow 2TPN^+ + H_2N_2O_2 + H_2O \qquad (5)$$

It is a pleasure to acknowledge the technical assistance of Mr. R. C. GREGOIRE.

Research Laboratory, Linde Co., Division of Union Carbide Corporation, Tonawanda, N.Y. (U.S.A.)

H. R. SCHREINER
A. P. RINFRET

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Cholinesterases in human serum

Kalow, Genest and Staron[1] commented on the wide range of published values for the Michaelis constant of benzoylcholine hydrolysis, but made no attempt to reconcile the various estimates. Of those relating to human serum there are on the one hand values of about $5 \cdot 10^{-6}$ *M* reported by Kalow[2] and confirmed by his later work, and on the other[3,4] values of about $2.5 \cdot 10^{-3}$ *M*. Whereas Kalow used a spectrophotometric method of estimating enzyme activity[2], others have used the Warburg manometric method, which is limited in its application to much higher substrate concentrations. It seemed possible that Kalow's enzyme was different from that described by others (*e.g.* Bergmann and Segal[3], Augustinsson[4] or Myers[5]). A re-examination of the problem, using a wide range of substrate concentrations and a number of inhibitors, has shown that this indeed appears to be the explanation for the discrepancy.

While this work was in progress, Heilbronn[6] demonstrated the presence in Harvard Fraction IV-6-3 from human plasma of two enzymes capable of hydrolysing butyrylcholine.

All specimens of serum were examined to ensure that the Dibucaine Number[7] was about 80, thus avoiding the "atypical esterase" of Kalow. Benzoylcholine hydrolysis was measured spectrophotometrically in 0.067 *M* phosphate buffer, pH 7.4, at 25°, using substrate concentrations up to 10^{-4} *M* (*cf.* ref. 2) and a final serum dilution of 1:200, and hydrolysis of both benzoyl- and acetylcholine at and above 10^{-4} *M* was measured by a continuous titration method in 0.154 *M* NaCl at pH 7.4 and 25°, using a final serum dilution of 1:100. Where comparison was possible, the two methods agreed within 15 %, and the spectrophotometric data were adjusted to match the continuous titration data. It is known that alterations in salt concentration have a relatively small effect on pseudocholinesterases[5], and it was therefore assumed that no serious error arose on this account, nor from variations in serum dilution noted.

Since the temperature used by Kalow was about 25°, but that used by others was 38°, this temperature difference might, at least in part, have accounted for the discrepancy; however, varying the temperature in the spectrophotometric method from 25° to 35° had no effect on the Michaelis constant.

Several specimens of serum were then examined to find the relationship between benzoylcholine concentration and enzyme activity. Fig. 1 shows some of the curves obtained. In Figs. 1a and 1b there are points of minimum activity in the region of 10^{-3} *M*. The most probable explanation is that this is due to the presence of two enzymes. Consequently when curves such as Fig. 1c are obtained (as also Kalow *et al.*[1], Fig. 6) it seems reasonable to interpret these too as showing the presence of two enzymes, and not as one enzyme incompletely inhibited by excess substrate[1]. It is noteworthy that over the range of substrate concentration usable in the Warburg apparatus (log[Benzoylcholine]-2.7 and above) the relationship between concentration and activity is variable. Bergmann and Segal[3], who merely published a value for the Michaelis constant, may have used a specimen with properties similar to those of Fig. 1a; Augustinsson[4] gave data indicating a flat peak of optimum activity,

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Abbreviations: TEPP, tetraethylpyrophosphate; DPDA, NN′-diisopropylphosphorodiamidic anhydride.

rather like Fig. 1b; and, as far as can be deduced from his comments, Myers[8] may have used a specimen with properties like those of Fig. 1c.

With some of these specimens, the hydrolysis of acetylcholine was also examined. Fig. 2 shows that plots of v against $v/(S)$ were not linear, but convex towards the origin. Hofstee[9] has pointed out that this signifies the presence of two (or more) enzymes acting independently on the same substrate; and such data were part of Heilbronn's[6] evidence that human serum contains two enzymes acting on butyrylcholine. The data fit the assumption of two enzymes with simple Michaelis-Menten kinetics, one acting at maximum velocity at 10^{-4} *M*.

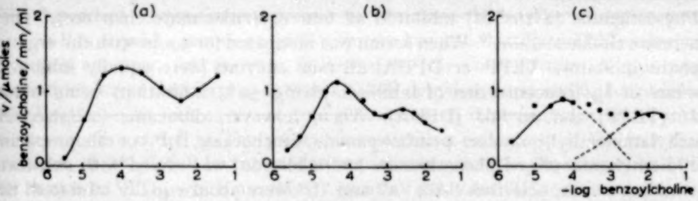

Fig. 1. Relationship between benzoylcholine concentration and enzyme activity in various specimens of human serum.

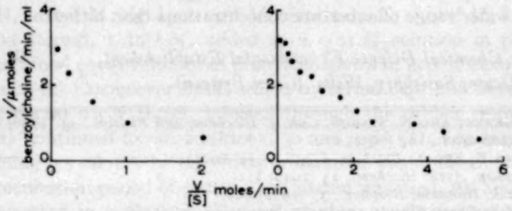

Fig. 2. Relationship between acetylcholine concentration and enzyme activity in specimens of human serum. Data transformed by Hofstee's[9] method.

We have, therefore, four enzyme activities, which may briefly be denoted as follows:

Activity "A", hydrolyses acetylcholine at high substrate concentrations, simple Michaelis-Menten kinetics, *Km* about 10^{-3} *M*, practically inactive at 10^{-4} *M* and nearly maximally active at $2 \cdot 10^{-2}$ *M*.

Activity "a", hydrolyses acetylcholine at low substrate concentrations, assumed to be maximally active at 10^{-4} *M*.

Activity "B", hydrolyses benzoylcholine at high substrate concentrations, *Km* about 10^{-3} *M*, and sometimes inhibited by excess substrate within the range studied. Assumed to be inactive at 10^{-4} *M*, and maximally active or nearly so at $2 \cdot 10^{-2}$ *M*.

Activity "b", hydrolyses benzoylcholine at low substrate concentrations, *Km* about 10^{-5} *M* and inhibited by excess substrate. Assumed to be optimally active at 10^{-4} *M* and inactive at $2 \cdot 10^{-2}$ *M*.

On the other hand, there might be only two enzymes; some studies with inhibitors were therefore carried out in an attempt to define the number of enzymes present. It follows from the above definitions that activities "B" and "b" can be measured at $2 \cdot 10^{-2}\,M$ and $10^{-4}\,M$ benzoylcholine respectively without further correction; that activity "a" can be measured at $10^{-4}\,M$ acetylcholine, and that if data obtained at this concentration are subtracted from those obtained at $2 \cdot 10^{-2}\,M$ a measure of activity "A" is given.

Physostigmine ($2 \cdot 10^{-6}\,M$) inhibited all four activities more than 80%. They are therefore cholinesterases[10]. When serum was incubated for 5 min with the organophosphate inhibitors TEPP or DPDA, all four enzymes were equally inhibited, the values of I_{50} (concentration of inhibitor giving 50% inhibition) being about $10^{-9}\,M$ (TEPP) and $10^{-4}\,M$ (DPDA). When, however, dibucaine [2-butoxy-N-(2′-diethylaminoethyl)quinoline-4-carboxyamide; Cinchocaine, B.P.] or ethopropazine [10-(2′-diethylaminopropyl)phenothiazine hydrochloride] was mixed with substrate before adding serum, activities "A", "a" and "b" were about equally inhibited, the I_{50} values being $2 \cdot 10^{-6}\,M$ (dibucaine) and $2 \cdot 10^{-7}\,M$ (ethopropazine). Activity "B" was much less sensitive, the values of I_{50} being $10^{-3}\,M$ and $2 \cdot 10^{-4}\,M$, respectively. The only reasonable explanation for this is that "B" is a separate enzyme, and cannot be associated with either "A" or "a". Since it is highly improbable that both "A" and "a" can be attributed to one enzyme, one of them may be associated with "b" to describe a second activity, leaving the other to describe a third.

This evidence of the presence of three cholinesterases in human serum, together with findings mentioned by HEILBRONN[6] that only two peaks of activity have been found in fractions separated by electrophoresis, suggests the need to examine such fractions with a wider range of substrate concentrations than hitherto.

War Department, Chemical Defence Experimental Establishment, W. K. BERRY
Porton Down, Salisbury, Wilts. (Great Britain)

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An effect of enzymic reduction of steroids on triphosphopyridine nucleotide-dependent glucose 6-phosphate oxidation

Mammalian liver possesses two series of enzymes, one soluble and the other microsomal, which catalyze the reduction of the α–β unsaturated A ring of many Δ^4-3-ketosteroids to form the corresponding saturated 3-ketosteroids. These reductases are characterized by a very high degree of substrate specificity and an obligatory requirement for TPNH as the reducing agent. Once formed, the saturated 3-keto-

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Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; G-6-P, glucose 6-phosphate.

steroids can be further reduced by TPNH (or DPNH) in the presence of a soluble 3α-hydroxysteroid dehydrogenase present in liver[1,2], so that two moles of TPNH are oxidized during the reduction of one mole of steroid. This suggested that steroids could stimulate reactions that require TPN if both the steroid reductases and the TPN-dependent enzymes were present in the same tissue. The oxidative pathway for G-6-P is a good example of such a system because it utilizes TPN exclusively as hydrogen acceptor, and its rate is limited by the concentration of this pyridine nucleotide[3]. Liver contains the enzymes, G-6-P dehydrogenase and 6-phosphogluconic dehydrogenase, as well as the steroid reductases, so it presented a model in which to show such a coupling. The present investigation makes use of the fact that the oxidation of [^{14}C]G-6-P by the oxidative pathway results in the conversion of C-1 of the hexose to $^{14}CO_2$, which provides a sensitive assay for this reaction sequence.

Homogenates were prepared in 3 volumes of cold 0.25 *M* sucrose with a Potter-Elvehjem homogenizer. The soluble fraction which contained both the steroid reductases (5β) and the dehydrogenases of the oxidative pathway was obtained by centrifuging this homogenate for 45 min at 100,000 × *g* and dialyzing the supernatant against distilled water for 18 h at 5°. Microsomes were prepared by the method of HOGEBOOM[4].

Incubations were carried out at 37° with constant shaking. The reaction mixture consisted of 1.5 ml of the above dialysate or 2 ml of crude homogenate with the following additions: [1-^{14}C]G-6-P, $1 \cdot 10^{-4}$ *M*, specific activity 16,200 counts/min/μmole; TPN, $2 \cdot 10^{-5}$ *M*; $MgCl_2$, $2 \cdot 10^{-3}$ *M*; nicotinamide, $1 \cdot 10^{-3}$ *M*; Tris buffer pH 7.4, 0.02 *M*; and steroid, $1 \cdot 10^{-4}$ *M*, added as a 0.01 *M* solution in propylene glycol. Controls contained equal volumes of propylene glycol in lieu of steroid. The reactions were run in 25-ml Erlenmeyer flasks sealed by serum caps and were stopped by the addition of 0.4 ml 0.5 *N* H_2SO_4 injected through the rubber stoppers after which shaking was continued for an additional 30 min; and CO_2 was trapped in a center well containing 0.5 ml 5 % LiOH. $^{14}CO_2$ production was approximately linear up to 1 h, so an incubation period of either 20 or 30 min was used. An aliquot of the alkali trap was counted in a Nuclear micromil window gas-flow counter, and duplicate experiments agreed within 10 %.

The steroid reductase (5β) and 3α hydroxysteroid dehydrogenase activities of the liver preparations were assessed as described previously[1,5]. The activity of 6-phosphogluconic dehydrogenase was determined by increase in absorbancy at 340 mμ in a Beckman Model DU spectrophotometer when the dialyzed soluble rat-liver fraction was mixed with 6-phosphogluconate and TPN at room temperature.

The liberation of $^{14}CO_2$ from [1-^{14}C]G-6-P by dialyzed soluble supernatant of rat liver was enhanced by the addition of various reducible steroids (Table I). An effect was observed with steroid concentrations as low as $5 \cdot 10^{-6}$ *M*.

When the steroid reductase and 3α hydroxysteroid dehydrogenase activities of the rat-liver fraction were assayed, it was found that those steroids exhibiting a stimulatory effect were reduced by the preparation*. It is of interest that tetrahydrocortisone, which has no group reducible by this preparation, did not stimulate G-6-P oxidation.

In other experiments it was found that the addition of rat-liver microsomes alone stimulated G-6-P oxidation presumably as a result of their endogenous "TPNH oxidase" activity[6]. If, as appeared probable, the steroid stimulated G-6-P oxidation by increasing the steady-state concentration of TPN, the stimulation should not have been observed when the TPN concentration was not rate-limiting. Indeed, when experiments were done with concentrations of TPN in excess of $5 \cdot 10^{-4}$ M, steroid addition had no effect.

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* We are indebted to Dr. J. MCGUIRE for these determinations.

TABLE I

EFFECTS OF STEROID ON G-6-P OXIDATION

Addition	*G-6-P oxidized (μmole)*	*Stimulation (%)*
None	0.042	0
Cortisone	0.077	83
Androstene-3,17-dione	0.071	69
Cortisol	0.059	40
Progesterone	0.054	29
Testosterone	0.052	24
Deoxycorticosterone	0.050	19
Δ^1-cortisol	0.049	17
Dihydrocortisone	0.378	800
Dihydrocortisol	0.282	570
Tetrahydrocortisone	0.045	7
Estradiol	0.035	−17

Furthermore, in a spectrophotometric study of 6-phosphogluconic dehydrogenase, TPNH did not accumulate as rapidly when steroid was added (Fig. 1), which is consistent with an increased rate of pyridine nucleotide reoxidation coupled to steroid reduction.

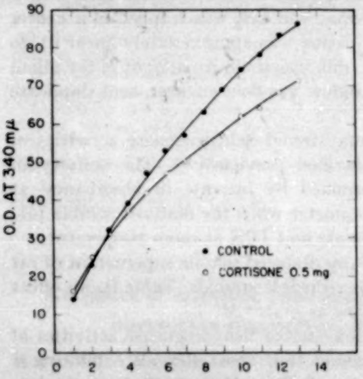

Fig. 1. Oxidation of 6-phosphogluconate by soluble supernatant of rat liver: effect of cortisone on TPNH accumulation. 2.5 ml of reaction mixture contained 0.1 ml soluble rat-liver supernatant; 0.02 M tris(hydroxymethyl)aminomethane buffer, pH 7.4; 6-phosphogluconate, $4 \cdot 10^{-4}$ M; $MgCl_2$, $1 \cdot 10^{-3}$; and TPN, $4 \cdot 10^{-5}$ M.

Homogenates of rat kidney and spleen were studied under similar conditions, and although each showed significant production of $^{14}CO_2$ from [1-^{14}C]G-6-P, neither was stimulated by the addition of steroids. These tissues are devoid of steroid reductase activity[1].

Although the data above show that various steroids could control the rate of G-6-P oxidation in a somewhat purified preparation, it was of interest to see if such effects could be demonstrated under more nearly physiological conditions. When crude homogenates were examined, $^{14}CO_2$ production from [1-^{14}C]G-6-P was stimulated by the addition of cortisone to the incubation mixture. In Fig. 2 the degree of stimulation of $^{14}CO_2$ production by cortisone is shown as a function of the length of incubation using a crude homogenate of rat liver. Similar results were obtained with liver slices when [1-^{14}C]glucose was used as substrate.

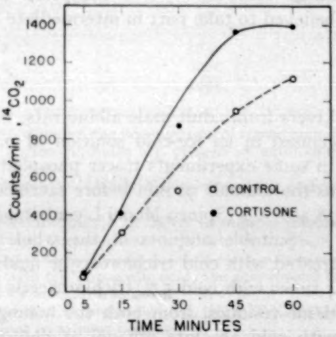

Fig. 2. Effect of cortisone on $^{14}CO_2$ production from [1-^{14}C]glucose by rat-liver homogenate. 5 ml reaction mixture contained 3 ml homogenate of rat liver, 0.02 M phosphate buffer, pH 7.4; DPN and TPN, $1 \cdot 10^{-4}$ M; $MgCl_2$, $1 \cdot 10^{-3}$ M; nicotinamide, $1 \cdot 10^{-2}$; ATP, $1 \cdot 10^{-3}$ M; and [1-^{14}C]glucose, $3 \cdot 10^{-3}$ M, specific activity, 20,000 counts/min/μmole.

It is important to note that G-6-P oxidation is a model reaction and that there may either be other TPN-dependent systems or reactions inhibited by TPNH which are more responsive to small changes in either the absolute quantity of TPN or the TPN/TPNH ratio. If there are, in fact, such reactions, they should display a more striking response to steroid than that observed here. Furthermore, if an enzyme were present to catalyze the re-introduction of the double bond using a hydrogen acceptor other than TPN, it would be possible for small quantities of steroid to provide an efficient mechanism for oxidation of TPNH. Such an enzyme, however, has not been found in animal tissues[7]. The present results, however, may provide an explanation for some of the effects of the steroid hormones *in vitro*.

National Institute of Arthritis and Metabolic Diseases,
National Institutes of Health, Public Health Service,
Department of Health, Education, and Welfare,
Bethesda, Md. (U.S.A.)

K. LEMONE YIELDING
GORDON M. TOMKINS

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Observations on the effect of ethanol on rat-liver ribonucleoprotein previously exposed to trichloracetic acid

In a previous paper[1] it was observed that cold absolute ethanol dissolved a part of the liver RNA of the whole homogenate or of the cytoplasmic fraction which has been previously precipitated and washed with cold dilute trichloroacetic acid. This study has now been extended to the supernatant fraction obtained by centrifugation of a liver homogenate at 78,400 × *g*. This fraction is known to contain ribonucleic acids which function as acceptors of enzymically activated amino acids[2] and are believed to take part in intermediate stages of protein synthesis[3].

METHODS

Livers from adult male albino rats, which had been fasted for 18–20 h, were homogenised in an ice-cold solution of 0.25 M sucrose containing 0.00031 M $KHCO_3$. In some experiments tracer phosphate (0.5 mC) was administered intraperitoneally to the animals 90 min before sacrifice. The homogenate was centrifuged for 3 h at 78,400 × *g* (Spinco Model L centrifuge, rotor No. 30) to give a supernatant fraction.

Suitable aliquots of the whole homogenate and supernatant fraction were treated with cold trichloroacetic acid at a final concentration of 2.5 % and washed 4 times with cold 5 % trichloroacetic acid. One set of acid-precipitated and washed tissue residues, from both the homogenate and supernatant fraction, were treated with cold absolute ethanol as described previously[1] and washed twice with cold absolute ethanol (Procedure I, Table I). The pooled alcohol extracts and washings were evaporated to dryness *in vacuo* and washed successively with large volumes of ether, petroleum ether, chloroform, and a mixture of chloroform–methanol (2:1, v/v). The residue was hydrolysed with 1 N KOH at room temperature for 18–20 h, neutralised by the addition of 3 N $HClO_4$ and the insoluble $KClO_4$ separated by centrifugation. The centrifugate was saved for analysis.

The second set of samples was treated and washed with trichloroacetic acid, as were the samples in Procedure I, but was not further treated with ethanol (Procedure II, Table I). The nucleic acids, from both the ethanol-treated and untreated tissue residues, were extracted with 3 % $HClO_4$ at 80–85° for 30 min[4].

Ribose was determined on aliquots of all the extracts by the method of BROWN[5]. In order to eliminate anomalous variation in absorbancy, all extracts, irrespective of prior treatment, were heated with 3 % $HClO_4$ for 15 min at 100° and the absorbancy at 260 mμ was determined.

In experiments in which tracer phosphate was administered to rats, the supernatant fraction was divided into two portions and treated according to Procedures I and II (as described above). From these washed tissue residues sodium nucleates were isolated by extraction with buffered salt solution according to the method of SIEKEVITZ AND PALADE[6]. Any lipid contaminants present in the nucleates were removed by successive washings with an ethanol–ether mixture (3:1, v/v), 95 % ethanol and finally ether. The dry pellet was hydrolysed with 0.5 N KOH to furnish a mixture of 2′,3′-ribonucleotides[6].

The pooled ethanol extract and washings, obtained by Procedure I in the tracer

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Abbreviation: RNA, ribonucleic acid.

TABLE I

EFFECT OF ABSOLUTE ETHANOL ON THE RNA CONTENT AND SPECIFIC ACTIVITY OF RNA PHOSPHORUS OF RAT-LIVER HOMOGENATE AND SUPERNATANT FRACTION PREVIOUSLY TREATED WITH TRICHLOROACETIC ACID

	*Ribose** (*μg/g fresh liver*)	*Absorbancy*** (*units/g fresh liver*)	*Specific activity****
Homogenate			
Procedure I §	3440	148	—
Ethanol-soluble fraction	1304	60	—
Procedure II §§	4800	191	—
Supernatant fraction			
Procedure I §	318	15	1851
Ethanol-soluble fraction	222	11	6660
Procedure II §§	510	22	2995

* The estimated value for ribose has been doubled to allow for the non-reaction of ribose bound to pyrimidines; it is assumed that pyrimidine and purine ribosides are present in equimolar amounts. Values are mean of five experiments.

** Absorbancy units = observed absorbancy at 260 mμ × volume. Values are mean of two experiments.

*** The specific activities were calculated as counts/min/μmole of RNA or ethanol-soluble polyribonucleotide phosphorus. Values are mean of two experiments in which only specific activities were determined.

§ Homogenate or supernatant fraction was treated with trichloroacetic acid; the acid-insoluble residue was washed with trichloroacetic acid followed by ethanol treatment.

§§ Homogenate or supernatant fraction was treated with trichloroacetic acid; the acid-insoluble residue was washed with trichloroacetic acid and ethanol treatment omitted.

experiments, were processed in the same manner as described in the previous section. However, the alkaline digest, prior to neutralisation with $HClO_4$, was again repeatedly extracted with petroleum ether to ensure complete removal of phospholipid material.

In each case, the mixture of 2′,3′-ribonucleotides was adsorbed on a Norit A column, washed with a large excess of non-radioactive inorganic phosphate, followed by distilled water, until the washings were free of phosphate. The nucleotides were eluted from the columns with an ammonia–acetone–water mixture[7]. Solutions containing the eluted mixture of nucleotides were wet-ashed with H_2SO_4 and HNO_3 and the inorganic phosphate precipitated with a magnesia mixture[8]. The $MgNH_4PO_4$ was dissolved in 0.5 N HCl and aliquots were used for phosphorus estimation[9] and measurement of radioactivity.

RESULTS

The data clearly demonstrate that cold absolute ethanol dissolved 25 to 30% of the total amount of RNA present in the liver homogenate which had been pretreated with and washed with cold dilute trichloroacetic acid. This finding confirms those of the previous report[1], in which, in addition, evidence was presented to suggest that the RNA susceptible to the solubilising action of ethanol appeared to be localized in the cytoplasmic fraction. It is also apparent from Table I that about 40% of the RNA present in the supernatant factor appears in the ethanol phase after precipitation with trichloroacetic acid, washing and treatment with ethanol. The specific activity of ethanol-insoluble, RNA-phosphorus (Procedure I, Table I) is 37% lower than that isolated by Procedure II, in which ethanol treatment was omitted. In contrast, the specific activity of the alcohol-soluble polyribonucleotide phosphorus is 2 to 4 times higher than those of the two RNA preparations isolated by Procedures I and II. Thus, it appears that the ethanol-soluble polyribonucleotide could be distinguished from the rest of the macromolecule by its higher rate of ^{32}P incorporation.

It has been suggested by SIEKEVITZ AND PALADE[6] that the high internal turnover of RNA associated with soluble ribonucleoprotein, obtained by prolonged centrifugation at 105,000 × *g* of the non-microsomal supernatant fraction, may be related to its function as an acceptor of activated amino acids. It is possible that the ethanol-soluble polyribonucleotide of the supernatant fraction, which exhibited a high rate of ^{32}P incorporation, may serve as a tool to examine some aspects of the above concept.

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Department of Pharmacology,
Yale University School of Medicine,
New Haven, Conn. (U.S.A.)
P. R. VENKATARAMAN

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Phage-cell binding in external electric fields and in viscous media

The rate-controlling step in the initial binding of bacteriophages to their host cells is the diffusion of virus to the cell surface. Measured attachment rates[1–4] agree closely with those calculated from SMOLUCHOWSKI theory[5], indicating that the efficiency of reaction per encounter is close to unity[*]. If by subjecting the system to appropriate force fields, virus and cell can achieve differential motion above that resulting from diffusion alone, then the encounter frequency, and hence the reaction rate, might be increased. Conversely, since the diffusion constant varies inversely with the viscosity, the rate of attachment should be decreased if the viscosity of the suspending medium is raised. Experiments are reported here in which both of these conditions were fulfilled, using the system T2 phage-*Escherichia coli* B.

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[*] See L. J. TOLMACH[6] for a brief discussion of the distinction between collision and encounter efficiency. A statement appearing in a recent publication[7], to the effect that this article questioned the conclusion that the reaction is very efficient, is in error.

Attachment in an external electric field

Advantage was taken of the different electrophoretic mobilities of cell and virus. Exponentially growing nutrient-broth cultures of B were killed by heating for 1 h at 63°, sedimented, and resuspended at about $2 \cdot 10^8$/ml in 0.067 *M* Na_2HPO_4–KH_2PO_4 buffer, pH 6.8. 0.1 ml of activated[8] T2, at about 10^4/ml in nutrient broth containing 0.5 % NaCl, was added to 0.9 ml of the cell suspension and the mixture quickly transferred to one arm of a 3 mm (internal dia.) U-tube immersed in a water bath at —3°. The field, with voltage monitored, was applied for 5 min. A 0.1-ml aliquot was rapidly removed from the tube with a chilled syringe and assayed for phage[9]. By using heat-killed cells a centrifugation step was obviated, thereby reducing the elapsed time between sampling and plating to 0.3–0.4 min. Tests showed that attachment was reliably measured by this procedure. Loss of neither phage viability nor cellular binding capacity was detected when either component individually underwent sojourns in the attachment tube with field applied.

Applied fields yielding various potential gradients resulted in variable, inhomogeneous heating within the reaction tube, arising from the large currents flowing in the highly conducting medium[3]. Accordingly, a standard gradient of 44 V cm^{-1} was adopted, and the direction of the field was reversed at specified frequencies with a motor-driven commutator. Thereby, variations of effective mobility were achieved under conditions of constant current flow: at low frequencies the system was subjected to essentially d.c. fields, while at sufficiently high frequencies no significant cell migration would occur, the attachment rate being the same as that in the absence of an external field, at the attained temperature. A small thermister inserted in the U-tube indicated temperatures near 15° at 20 mA, 44 V cm^{-1}. Observation with an oscilloscope revealed a clean square wave up to about 70 sec^{-1}.

Fig. 1 shows the dependence of attachment rate constant on frequency. Exact correspondence between the rate at 70 sec^{-1} and that measured with no applied field (at 15°) is questionable because of the absence of temperature uniformity within the reaction tube. Lacking an adequate quantitative theory[2] to apply to these data, we may consider that migration of the cell causes it to sweep out volumes of solution in which it encounters virus particles. Then, to the rate constant, *k*, calculated from the (time-independent) SMOLUCHOWSKI equation, is added the contribution from additional encounters experienced during these excursions:

$$k = 4\pi D_{\mathrm{virus}}\, r_{\mathrm{cell}} + \pi r^2_{\mathrm{cell}}\, v,$$

where v is the velocity of the cell relative to the virus. Adopting $8.2 \cdot 10^{-5}$ cm for the effective radius of the cell, $3.0 \cdot 10^{-8}$ cm^2 sec^{-1} for the diffusion constant of T2[10], and, respectively, $-3.7 \cdot 10^{-4}$ and $-0.6 \cdot 10^{-4}$ cm^2 V^{-1} sec^{-1} for the mobilities of B[11] and T2[12], the calculated ratio of rate constants at low and high frequency in a gradient of 44 V cm^{-1} is about 4 times the ratio of 2.5 measured. In view of the possibility that suppression was incomplete at 70 sec^{-1}, and the crudeness of the model, the agreement is acceptable. With the relatively small increase in rate constant obtained, attachment rates greater than those predicted by the SMOLUCHOWSKI equation were not demonstrable; however, a virus-cell system in which binding occurs at lower salt concentrations might allow employment of larger potential gradients, with consequent achievement of greater increases in rate.

The effects of field reversal may be accounted for by assuming the cell to sweep out a cylindrical volume in which it encounters additional virus unless it reverses its direction so frequently that bulk virus concentration cannot be reestablished between excursions. Thus the frequency at which suppression occurs depends on the virus diffusion constant. It may then be roughly estimated that the added number of encounters will be half of maximal when the time between excursions is such that the root mean square displacement of virus due to diffusion (normal to the surface of the cylinder) is half the radius of the cylinder. The EINSTEIN equation yields a reversal frequency of 35 sec^{-1} for half-maximal effect, in reasonable agreement with the measured value of about 10 sec^{-1} (Fig. 1).

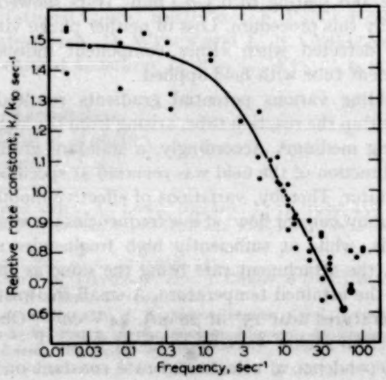

Fig. 1. Variation of attachment rate constant with frequency of field alternation. Each point represents a separate measurement of the amount of unattached phage remaining after the attachment reaction was terminated. Data from two separate runs, carried out under slightly different conditions, are included. They are normalized at 10 sec^{-1} (the star on the figure). The values obtained at frequencies greater than 70 sec^{-1} are not reliable because arcing occurred.

These results demonstrate the utility of employing external electric fields in the study and control of certain diffusion-controlled reactions. It should be possible, for example, to make independent determinations of the relative diffusion constants of reacting charged particles.

Attachment in viscous media

Early experiments[13] failed to show an inverse relation between attachment rate and viscosity. To investigate this anomaly[4], the rate of attachment of T2 to *E. coli* B was measured in 0.067 *M* phosphate buffer made viscous by addition of solid sucrose. A killed-cell suspension was prepared as in the previous experiments, added to the sucrose-phosphate solutions at about 10^8/ml, and equilibrated at 37°. Aliquots of T2 were added at about 10^5/ml, and samples were withdrawn periodically and plated for plaques, centrifugation of the viscous solutions being unnecessary.

Table I shows that over a 5-fold range of viscosity the attachment rate constant is inversely proportional to the viscosity, as theory predicts.

Because of the steric specificity of the attaching surfaces, it has been questioned that the initial binding of phage to host cells is diffusion-controlled[14,15], despite the small temperature coefficient of the reaction[16,17] and the aforementioned close correspondence of measured with calculated rate constants. Were the reaction not diffusion-limited, it would not be expected that altering the encounter frequency, as was done in two different ways in these experiments, would change the reaction rate.

TABLE I

EFFECT OF VISCOSITY ON THE REACTION RATE CONSTANT FOR ATTACHMENT OF T2 TO *E. coli* B AT 37°

Sucrose concentration (%)	*Viscosity η (centipoises)*	*Relative values*		
		$1/\eta$	*Rate constant* *Expt. 1*	*Expt. 2*
0	0.69	1	1	1
20	1.27	0.54	0.53	0.55
40	3.57	0.19	0.22	0.16

Helpful discussions of this work were held with Drs. L. S. LERMAN (who suggested alternating the field polarity) and T. T. PUCK. This study was aided by a grant from the Damon Runyon Fund.

L. J. TOLMACH*

*Department of Biophysics**, University of Colorado Medical Center,*
Denver, Colo. (U.S.A.)

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* Present address: Department of Radiology, Washington University School of Medicine, St. Louis, Mo. (U.S.A.).

** Contribution No. 100.

The detection of terminal nucleoside residues in polyribonucleotides

Some ribonucleic acids are terminated at one end by a nucleoside residue linked through its 5-′hydroxyl group to the rest of the polynucleotide chain. The polyribonucleotides formed by polynucleotide phosphorylase[2] and the "soluble" RNA fraction from mammalian tissues[3] and *Escherichia coli*[4] have terminal residues of this type. Oxidation by periodate of the 2′ and 3′ *cis*-hydroxyls of the terminal nucleoside residue yields a dialdehyde derivative of the nucleoside (nucleoside dialdehyde) without detaching this from the rest of the molecule[5]. The reactive aldehyde groups so produced offer the possibility of introducing a radioactive labelled group specifically into the terminal residue, thus facilitating the assay and identification of end residues of this type. For this purpose we have made a preliminary investigation of the reaction of ribonucleoside dialdehydes (and polynucleotides terminated by these residues) with thiosemicarbazide. An attempt was also made to use this approach to investigate the nature of the end group in two viral ribonucleic acids.

[^{35}S]Thiosemicarbazide was prepared from potassium [^{35}S]thiocyanate[6]. The latter was converted to the ammonium salt by passage through a column of Dowex 50-(NH$_4$)$^+$. 10 mg ammonium thiocyanate, 4 mg hydrazine hydrate and 4 µl water were heated with refluxing in a sealed capillary tube at 125° for 1 h. The thiosemicarbazide, which crystallized on cooling, was recrystallized from 50% (v/v) ethanol–water, dissolved in water and shaken with a small amount of anion exchanger (IRA 400-acetate) to remove traces of thiocyanate.

[^{35}S]Thiosemicarbazone derivatives of the 5′-phosphates of adenosine, guanosine, cytidine and uridine dialdehydes were prepared on filter-paper strips in the following way. An aqueous solution of the 5′-nucleotide (0.025 *M*) was reacted with sodium metaperiodate (0.07 *M*) at pH 4 for 1 h at 0° in the dark to yield the 2′, 3′-dialdehyde. 30 µl of the reaction mixture was placed in a band on Whatman 3 MM filter paper and subjected to electrophoresis in 0.1 *M* acetate buffer, pH 5, until the iodate and periodate had migrated ahead of the nucleotide towards the anode (50 min at 20 V/cm). A band of excess [^{35}S]thiosemicarbazide was placed between the oxidized nucleotide and the periodate band. Electrophoresis was continued allowing the oxidized nucleotide to migrate through the thiosemicarbazide band, which does not move. During the mixing of the two substances on the paper the dialdehyde reacts quantitatively with the thiosemicarbazide. The mononucleotide thiosemicarbazone derivative emerged and continued to migrate as a single band. This was verified by comparing the positions of the nucleotide derivative detected by its u.v. absorption[7] and of the thiosemicarbazide moiety by autoradiography. Each of the nucleotides was converted to a single u.v.-absorbing, radioactive thiosemicarbazone derivative. This was eluted in water. The excess thiosemicarbazide, being uncharged, remained stationary. The same procedure was used to obtain dialdehyde [^{35}S]thiosemicarbazones of the dinucleoside monophosphates GpC, GpU, ApC and ApU (prepared by dephosphorylation of the corresponding dinucleotides isolated from pancreatic ribonuclease hydrolysates of RNA[8]) and of CpU (prepared synthetically by the action of pancreatic ribonuclease on cytidine 2′,3′ cyclic phosphate and uridine[1]).

Abbreviations: RNA, ribonucleic acid; GpC, GpU, ApC and ApU, conventions adopted by HEPPEL *et al.*[1]; poly AGCU, biosynthetic polymer of adenylic, guanylic, cytidylic and uridylic acids.

The 2′- and 3′-phosphates of adenosine, guanosine, cytidine, and uridine did not react with thiosemicarbazide under these conditions. Cytidine dialdehyde thiosemicarbazone was prepared in a similar manner using electrophoresis at pH 3.5 where the cytidine dialdehyde and its thiosemicarbazone migrate towards the cathode. The corresponding adenosine and uridine derivatives were obtained by reaction of the dialdehydes with thiosemicarbazide at pH 5 in aqueous solution and were crystallized from water.

All the [^{35}S]thiosemicarbazone derivatives migrated as single u.v.-absorbing and radioactive substances on paper electrophoresis at pH 3.5 and 5, and on paper chromatography in isopropanol–acetic acid–water (70:5:25, v/v/v) (Solvent 1) and in ethanol–aqueous *M* ammonium acetate buffer, pH 5 (60:40, v/v) (Solvent 2). The absence of unreacted dialdehydes was shown by electrophoresis of the nucleoside and 5′-mononucleotide thiosemicarbazone derivatives in 0.05 *M* ammonium acetate, pH 5, containing 0.02 *M* sodium bisulphite. The dialdehydes form bisulphite–addition compounds which migrate towards the anode with approximately twice the mobilities of the nucleotides or their dialdehyde thiosemicarbazone derivatives.

The composition of the 5′-nucleotide dialdehyde thiosemicarbazones were determined by measurement of ^{35}S, and spectrophotometric estimation of the purine or pyrimidine after hydrolysis in 72 % (w/w) $HClO_4$ followed by paper chromatography in isopropanol–HCl–water[9]. The specific activity of the thiosemicarbazide used in the preparation was determined from its ^{35}S content and absorbancy at 235 mμ (taking ε as 14,000 at pH 7). Approximately 1 mole of thiosemicarbazide was found to have combined with 1 mole of nucleotide dialdehyde. BARRY *et al.*[10] have previously shown that 1 mole of thiosemicarbazide combines with 1 mole of dialdehyde in periodate-oxidized starch.

The dialdehyde thiosemicarbazones of the 5′-nucleotides were stable in the pH range 2–8 but above pH 8 slowly decomposed giving two unidentified S-containing substances running faster than the original derivatives in Solvent 2. The possibility of using alkaline hydrolysis of RNA labelled in this manner to identify terminal nucleoside residues was thus excluded. Consequently, we investigated the action of enzymes on the dialdehyde thiosemicarbazones of the 5′-nucleotides and dinucleoside monophosphates. The 5′-nucleotide dialdehyde thiosemicarbazones were converted by prostate phosphomonoesterase to the corresponding nucleoside thiosemicarbazone derivatives. They were not dephosphorylated by Russell viper venom at pH 8 although prolonged incubation at this pH resulted in some non-enzymic decomposition. The thiosemicarbazone derivatives of GpU and GpC were slowly hydrolysed at pH 8 by the diesterase of snake venom at a rate somewhat greater than the hydrolysis of the dinucleotides GpUp and GpCp but much slower than that of the dinucleoside monophosphates. Pancreatic ribonuclease, however, completely hydrolyzed CpU dialdehyde thiosemicarbazone to cytidine 3′-phosphate and uridine dialdehyde thiosemicarbazone (identified after chromatography in Solvent 1).

It is thus likely that a terminal nucleoside dialdehyde thiosemicarbazone residue in a polynucleotide will be liberated by ribonuclease (depending on the terminal sequence of the RNA) either as the nucleoside derivative or in a polynucleotide linked to one or more purine nucleotide residues. A preliminary application of the method for the characterization of such terminal sequences was made using poly AGUC formed by polynucleotide phosphorylase[11], and the RNA's of turnip yellow mosaic and tobacco mosaic viruses. Turnip yellow mosaic virus RNA was prepared according to MARKHAM AND SMITH[12] and tobacco mosaic virus RNA by the method of GIERER AND SCHRAMM[13]. $NaIO_4$ ($5 \cdot 10^{-4}$ *M*) was added to a solution of poly AGUC (2 mg/ml) or virus RNA (2–10 mg/ml) and the mixture left 10 min at 20°. This treatment was sufficient to oxidize the terminal nucleosides of poly AGUC although in some experiments higher concentrations of $NaIO_4$ were used. The polymer was precipitated by the addition of ethanol to 67 % (v/v), dissolved in 0.1 *M* NaCl containing 0.05 *M* sodium acetate buffer, pH 5 and reprecipitated with ethanol to remove periodate and iodate. The polymer was redissolved in NaCl–acetate buffer and allowed to react with [^{35}S]thiosemicarbazide ($1.5 \cdot 10^{-3}$ *M*) for 4 h at 20°. Under these conditions reaction with the thiosemicarbazide, determined by uptake of ^{35}S by the polymer was almost complete. The polymer was precipitated with ethanol and reprecipitated three times from NaCl–acetate buffer containing 0.05 *M* unlabelled thiosemicarbazide. It was finally reprecipitated from NaCl–acetate buffer and redissolved in water. The amount of thiosemicarbazide combined with the polymer was determined from measurement of its radioactivity and the total polymer concentration from the absorbancy at 260 mμ.

The poly AGUC was found to have taken up 1 mole thiosemicarbazide/57 moles nucleotide residues, while less than 1.5 % of this amount was taken up if the periodate treatment was omitted. The ratio ^{35}S/(absorbancy at 260 mμ) was unchanged after further incubation with unlabelled thiosemicarbazide and reprecipitation with ethanol.

Approximate estimation of the terminal nucleoside residues of the poly AGUC sample by measurement of the amounts of nucleoside liberated on alkaline hydrolysis gave 1 terminal nucleoside residue/30–40 nucleotide residues. The reason for this discrepancy was not determined; it may be due to a loss of low-molecular-weight polymer during the successive precipitations after labelling with thiosemicarbazide, or to other complications in the procedures.

The location of the [^{35}S]thiosemicarbazide in the terminal nucleoside residues is supported by the results of experiments in which the polynucleotide was digested with ribonuclease and the digest run on paper electrophoresis in 0.05 *M* ammonium acetate, pH 5. At this pH the purine and pyrimidine residues are almost uncharged and do not significantly contribute to the mobilities of nucleotides and polynucleotides. The nucleoside dialdehyde thiosemicarbazones remain stationary, while derivatives of type Pu.p.R, Pu.p.Pu.p.R, etc. [Pu denotes a purine nucleoside residue, R a nucleoside dialdehyde thiosemicarbazone residue, and p a phosphate] migrate towards the anode in order of increasing mobilities, but behind the mono- and polynucleotides. Autoradiography of the paper strips showed radioactive bands corresponding in position to substances of types R, Pu.p.R., Pu.p.Pu.p.R, and Pu.p.Pu.p.Pu.p.R which would be expected to be formed from a random polymer. The major u.v.-absorbing band of mono- and polynucleotides was non-radioactive.

The ribonucleic acid preparations from turnip yellow and tobacco mosaic viruses each took up about 1 mole [^{35}S]thiosemicarbazide/$0.4–1 \cdot 10^4$ moles nucleotide residues; however, the radioactivity did not have properties consistent with being attached to a terminal nucleoside residue. For example, varying the time of periodate oxidation showed that attachment of the thiosemicarbazide was dependent on an oxidation proceeding considerably slower than that of the terminal nucleosides of poly AGUC. Furthermore, after ribonuclease digestion of the ribonucleic acids the radioactive material did not have electrophoretic or chromatographic properties of the expected nucleoside or polynucleotide derivatives. The virus ribonucleic acids thus appear to have no terminal nucleoside residues since a specific attachment of less than one [^{35}S]thiosemicarbazide per RNA complement of the virus particle would have been detected.

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Agricultural Research Council Virus Research Unit, R. Dulbecco[*, **]
Huntingdon Road, Cambridge (Great Britain) J. D. Smith[*]

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[*] Present address: California Institute of Technology, Pasadena, Cal.

[**] Fellow of the Guggenheim Foundation and the recipient of a Fulbright Grant.

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Some enzymic actions on hydrouracil derivatives

In the course of work previously reported[1,2] it was desired to stimulate dihydrouracil or CβA utilization by attacking their derivatives with enzymes catalyzing nominally irreversible reactions, thereby removing the products from the equilibrium mixtures. It was necessary to assess the specificity of the relevant enzymes and, in one case, to devise a purification for 3′-nucleotidase because the reported procedure[3] failed in most respects in this laboratory.

For the following studies the derivatives of dihydrouracil were prepared as previously described[1,2]. These and other substrates were converted to the potassium salts and trace elements were removed by treatment with Dowex 50 (K form) or by extraction with dithizone. Absence of "heavy metals" was verified by the dithizone test. For the preparation of 3′-nucleotidase all operations were conducted at or near 0° and deionized distilled water was used unless otherwise specified.

The preparation of 3′-nucleotidase was as follows: 250 g commercial domestic (Oregon) rye-grass seed were moistened with water and allowed to germinate for

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Abbreviations: CβA, N-carbamoyl-β-alanine; ATP, adenosine 5′-triphosphate; CARP, carbamoyl-β-alanine ribotide; Tris, 2-hydroxymethyl-1,3-propandiol-2-ammonium chloride.

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2 days at 24°. The moist seed was homogenized (3 min in a Waring Blendor) with 500 ml iced water. The homogenate was then strained and the entrained liquid was expressed from the residue. The extract was centrifuged 15 min at 3900 × *g*. To the supernatant (Fraction A: 470 ml, 7.3 mg/ml protein, 15,400 units, specific activity 4.5) was added solid $(NH_4)_2SO_4$ to 3.6 *M*. The suspension was stirred 40 min and centrifuged 20 min at 3000 × *g*. The enzyme-containing fraction floated to the surface and was filtered onto a coarse paper. The dark residue was dispersed in 100 ml water and was dialyzed for 2 h in 8 l water and the dialysis was repeated once. The dialyzed solution was centrifuged 10 min at 3900 × *g* giving a clear, dark supernatant (Fraction B: 166 ml, 2.0 mg/ml protein, 8800 units, specific activity 26.5); concentration of residual $(NH_4)_2SO_4$, 0.028 *M*.

Solid $(NH_4)_2SO_4$ and 15 *M* NH_4OH was added to B to 1.7 *M* $(NH_4)_2SO_4$ and pH 8.0. The mixture was stirred 10 min and centrifuged 10 min at 3900 × *g*. 3.75 *M* $(NH_4)_2SO_4$, pH 8.0, was added to the supernatant to give a final $(NH_4)_2SO_4$ concentration of 3.0 *M*. After 10 min of stirring the precipitate was centrifuged 10 min at 3900 × *g* and was redissolved in 8 ml water, giving Fraction C (11 ml, 12.3 mg protein/ml, 5740 units, specific activity 42.5).

Fraction C was twice dialyzed for 10 h in 8 l water. After cooling the dialyzed solution to incipient ice formation, 0.35 volume 2-propanol at —30° was added. The mixture was stirred slowly for 10 min at —10° and centrifuged 10 min at 3400 ×*g*. 0.45 volume (referred to dialyzed C) of 2-propanol was added to the supernatant. After 10 min of stirring at — 10°, the suspension was centrifuged 10 min at 3400 × *g*. The residue was resuspended in 5 ml water and was dialyzed against 8 l water, giving Fraction D (6 ml, 8.6 mg protein/ml, 2800 units, specific activity 54).

Fraction D was less pigmented than the preceding fractions. Undialyzed fraction D was able to survive storage for 18 h at 24° without loss of activity. Contrary to the previous-described preparation[3], however, it is not stable for more than 3 min at temperatures above 60° nor to pH's less than 5.0. It contains extraneous phosphatases less than 5% as active as the nucleotidase at pH 7 and a ribonuclease activity (release of acid-soluble nucleotide) at pH 4.5 less than 20% that of the nucleotidase at pH 7. 3′-guanylate was attacked 97% as rapidly as 3′-adenylate in contrast to the previously published specificity. At no stage of the purification was either calcium phosphate gel or alumina Cγ a useful adsorbent.

A unit of enzyme changes 1 μmole substrate/h at 38°; specific activity is units/mg

TABLE I

ACTION OF 3′-NUCLEOTIDASE ON DIHYDROURACIL DERIVATIVES COMPARED TO THAT ON OTHER NUCLEOTIDES

Composition of assay mixture: 50 m*M* Na maleate, pH 6.95, 2 m*M* substrate, 2 units/ml enzyme; 5 min at 38°. Phosphate release was measured after stopping the reaction by addition of $HClO_4$ to 0.5 *M*.

Compound	*Relative rate*	*Compound*	*Relative rate*
3′-adenylate	1.000	3-′uridylate	0.524
3′-dihydrouridylate	0.194	5′-dihydrouridylate	0.104
3′-CARP	0.137	5′-uridylate	0.083

protein. Protein was determined by a biuret method[4] using dried casein as the standard. Phosphate was measured by the FISKE-SUBBAROW procedure[5].

Table I illustrates the action of 3′-nucleotidase on a number of dihydrouracil and CβA derivatives. The specificity of 3′-nucleotidase is not as strict as is that of 5′-nucleotidase.

Table II represents the action of 5′-nucleotidase[6] on a number of dihydrouracil nucleotides and derivatives. This virtually absolute discrimination between the dihydrouracil and CβA derivatives was the basis of experiments demonstrating the closure of the CβA moiety to give the nucleotidase-susceptible dihydrouracil 5′-ribotide described in a previous paper[2].

TABLE II

ACTION OF 5′-NUCLEOTIDASE ON DIHYDROURACIL DERIVATIVES COMPARED TO THAT ON OTHER NUCLEOTIDES

Composition of assay mixture: 50 m*M* Tris, pH 8.6, 2 m*M* substrate, 10 m*M* MgSO$_4$, 2 units/ml enzyme; 10 min at 38°. Phosphate release was measured after stopping the reaction by addition of HClO$_4$ to 0.5 *M*.

Compound	*Relative rate*	*Compound*	*Relative rate*
5′-adenylate	1.000	3′-dihydrouridylate	0.011
5′-dihydrouridylate	1.325	3′-CARP	0.000
5′-CARP	0.004	ATP	0.037
5′-uridylate	0.970	Ribose-5-phosphate	0.011
5′-cytidylate	0.752	3′-uridylate	0.016

Uridine hydrolase[7] was tested with the ribosides of dihydrouracil and CβA. These derivatives were completely inert to the enzyme. This agrees with previous observations on the reactivities of hydropyrimidine nucleosides[8].

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McIlvain Laboratories, Department of Medicine, L. C. MOKRASCH
University of Kansas Medical Center, Kansas City, Kan. (*U.S.A.*) S. GRISOLIA

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The photodecomposition of dinitrophenyl-amino acids*

It is recognized that a principle shortcoming in the estimation of protein N-terminal amino acids as their dinitrophenyl derivatives is the difficulty encountered in accurately evaluating DNP-amino acid losses occurring during hydrolysis and the subsequent operations required for quantitation[1]. In attempting to evaluate such losses employing DNP-[^{14}C]amino acids as internal standards, we observed a variable and often an extensive loss of radioactivity when the derivatives were not shielded from light. The necessity for avoiding photodecomposition of DNP-amino acids has been mentioned by a number of authors[2-4]. Akabori *et al.*[5] have reported on the rate of the photolytic decomposition and recently have shown that certain DNP-peptides[6] are also light sensitive. Similar observations have been reported for trinitrophenylated peptides[7]. However, the nature or the extent of the decomposition has received little attention.

The DNP derivatives of [2-^{14}C]glycine, [2-^{14}C]tyrosine, [3-^{14}C]serine, [1-^{14}C]-valine (Nuclear Chicago Corporation), [1-^{14}C]alanine and [1-^{14}C]glycine (Isotope Specialties) were prepared following the method of Rao and Sober[8]. The DNP-amino acids were not recrystallized but were purified by silicic acid chromatography[9] thus removing dinitrophenol and dinitroaniline.

Aliquots of the DNP derivatives in acetone solution were plated onto stainless-steel planchets and exposed to two different lighting conditions occurring in the laboratory. Fig. 1A demonstrates the rate of activity loss when the samples were exposed to light in the usual working area approximately 12 feet from outside windows. The loss was greatly accelerated when a parallel group of samples was exposed to daylight adjacent to an outside window but protected from direct sunlight

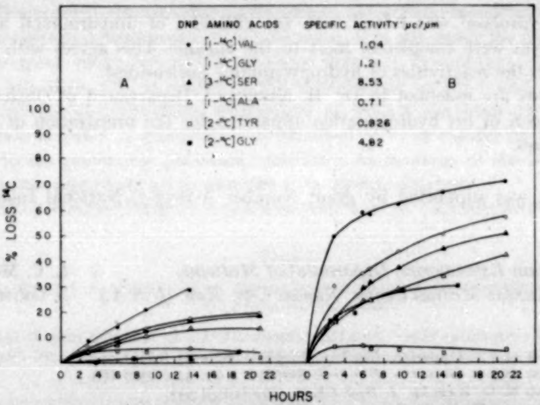

Fig. 1. Effect of light on the decomposition of DNP-[^{14}C]amino acids plated on stainless steel. A, compounds exposed to light 12 feet from window. 1B, compounds exposed to light 1 foot from window. (See text.)

Abbreviations: DNP-, dinitrophenyl.

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(Fig. 1B). Other experiments showed that the decomposition rate varied with the nature and intensity of illumination, the area of sample distribution and the nature of the supporting medium. A group of samples kept in total darkness failed to lose activity over the same period. A decline in the rate of loss, more prominent in Fig. 1B, is interpreted as the effect of decomposition products accumulating on the sample surface reducing the effective light intensity.

In other experiments, aliquots of the radioactive DNP derivatives were subjected to photodecomposition in Conway-Kirk diffusion flasks. The center wells of the flasks contained saturated $Ba(OH)_2$ or 1 *N* NaOH. Following exposure to daylight for periods of 48–72 h, the alkali trap was checked for radioactivity. The carboxyl-labeled compounds yielded radioactive CO_2 which was recovered directly as $BaCO_3$. On the other hand, DNP-[3-^{14}C]serine gave rise to volatile radioactive compounds which were trapped in NaOH and could be recovered as $BaCO_3$ only after persulfate oxidation. These results suggested that volatile acidic compounds are formed. Although DNP-[2-^{14}C]tyrosine did not lose radioactivity, chromatography of a sample which had been exposed to light demonstrated that DNP-[2-^{14}C]tyrosine decarboxylates to form radioactive DNP-tyramine (*vide infra*). Table I summarizes these experiments. Decarboxylation appears to be a principle but not the sole photolytic reaction.

TABLE I

RECOVERY OF RADIOACTIVITY FROM DNP-[^{14}C]AMINO ACIDS EXPOSED TO LIGHT IN CONWAY-KIRK DIFFUSION FLASKS

Added radioactivity was determined with unexposed derivatives plated on stainless steel at essentially zero thickness. Volatile radioactivity was counted as $BaCO_3$, not corrected for self absorption. All counts were corrected for coincidence loss.

DPN compound exposed	*Radioactivity* *Activity added* *10^3 counts/min*	 *CO_2 recovered as $BaCO_3$ from $BaOH_2$* *10^3 counts/min*	 *CO_2 recovered as $BaCO_3$ from 1 N NaOH after persulfate oxidation* *10^3 counts/min*
[1-^{14}C]alanine	64.0	29.4	a*
[1-^{14}C]glycine	126	48.5	a*
[1-^{14}C]valine	28.8	19.8	a*
[2-^{14}C]glycine	170	0.4	40.0
[3-^{14}C]serine	64.8	0.2	17.2
[2-^{14}C]tyrosine	30.0	0.0	0.5

* a not determined.

In another series of experiments the derivatives were spotted on strips of No. 1 Whatman paper and exposed to daylight and artificial light for up to 240 h to obtain maximum decomposition. The strips were subsequently chromatographed in the ascending system of BISERTE AND OSTEUX[10] along with unexposed control strips. These chromatographed strips were then scanned for radioactivity in an end-window counter adapted for manual strip counting using a slit width of 0.25 inch. Figs. 2A and 2B illustrate typical radiochromatograms. All exposed compounds yielded an increase in color intensity of the band at the solvent front as compared to unexposed samples. Only compounds containing radiocarbon at C-2 or C-3 exhibited a significant increase of radioactivity in this region. Unexposed samples of derivatives labeled at

C-2 or C-3 yielded some radio-activity in the solvent-front band. This band was shown to be due to decomposition occurring during normal handling operations as evidenced by the reappearance of a small radioactive peak at the solvent front when DNP-[2-^{14}C]glycine, purified by paper chromatography, was eluted and rechromatographed.

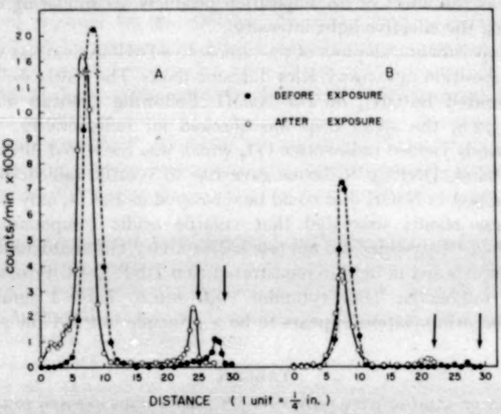

Fig. 2. Typical radiochromatograms of DNP-[^{14}C]amino acids before and after exposure to light. 2A, DNP-[2-^{14}C]glycine before and after exposure. Arrows indicate solvent front. The small peaks of low R_F also appear in radiochromatograms of DNP-[3-^{14}C]serine. Bands corresponding to these peaks are colorless and remain colorless when treated with ninhydrin. 2B, [1-^{14}C]glycine before and after exposure.

These observations and those cited above suggested that N-alkyl dinitroanilines result from photo-induced decarboxylation of DNP-amino acids. Hydrolysates of DNP-proteins subjected to chromatography using the ascending system of BISERTE AND OSTEUX yield a yellow band at the solvent front said to be dinitroaniline[11]. It appeared likely that N-alkyl dinitroanilines, which are not resolved from dinitroaniline in this system, might also be present. Accordingly, the "dinitroaniline" bands were eluted with chloroform from chromatograms of exposed DNP-[2-^{14}C]tyrosine, DNP-[2-^{14}C]-glycine, DNP-[1-^{14}C]glycine and DNP-[1-^{14}C]alanine and subjected to reverse-phase chromatography[12] in parallel with authentic DNP-tyramine, N-methyldinitroaniline and N-ethyldinitroaniline. DNP-tyramine was identified as the photodecomposition product of DNP-tyrosine and no dinitroaniline was evident with the reverse-phase system employed. While N-methyldinitroaniline and N-ethyldinitroaniline have an R_F identical with that of dinitroaniline, the compound obtained from exposed DNP-[2-^{14}C]glycine was radioactive whereas those obtained from DNP-[1-^{14}C]glycine and DNP-[1-^{14}C]alanine were nonradioactive. If dinitroaniline were a product of the decarboxylation of DNP-[^{14}C]amino acids, it should be nonradioactive. Since DNP-[^{14}C]tyramine was identified as a product formed from DNP-[2-^{14}C]tyrosine while dinitroaniline was not formed, the experiments indicate that N-methyl and N-ethyl-dinitroaniline are products formed from DNP-glycine and DNP-alanine respectively.

In summary the photodecomposition of DNP-amino acids yields CO_2, N-alkyl dinitroanilines and volatile acidic compounds of unknown structure. Photodecomposition may cause large and variable losses of DNP-amino acids unless exposure to light is avoided.

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Departments of Pediatrics and Physiological Chemistry, B. POLLARA
Research Laboratories of the Variety Club Heart Hospital, R. W. VON KORFF*
University of Minnesota, Minneapolis, Minn. (*U.S.A.*)

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* Established Investigator, Minnesota Heart Association.

Synthesis of serine from glycine in mitochondrial fragments

The conversion of two molecules of glycine to serine has been observed in the intact rat[1], In homogenates of turkey liver[2] and in a particulate fraction of rat liver[3]. Some of the intermediate steps in the synthesis have been studied in partially purified systems (see ref.[4]). Incidental to a search for an enzyme system capable of synthesizing δ-aminolevulinic acid, we observed that the entire complement of enzymes and cofactors required for the net synthesis of serine from glycine was present in a particulate fraction obtained by osmotic disruption of mitochondria.

The mitochondrial preparations were obtained from chicken livers by the conventional technique involving differential centrifugation in 0.3 M sucrose[5]. The preparation gave P/O ratios of 1.7–1.9 succinate and 2.3–2.6 with glutamate. The sub-mitochondrial particles were obtained by suspending the mitochondria in $1 \cdot 10^{-3} M$ tris(hydroxymethyl)aminomethane at pH 7.0 for 30 min at 0° and centrifuging at 100,000 × g for 15 min. The residue was suspended in an amount of 0.3 M sucrose equal to the original mitochondrial volume. The treatment results in disintegration of mitochondria and loss of approximately one-third of the protein in the supernatant.

For the assay, an aliquot of the enzyme preparation was incubated in a volume of 3.0 ml with $0.8–1.0 \cdot 10^{-2} M$ [2-^{14}C]glycine (18,650 counts/μmole), $8 \cdot 10^{-4} M$ pyridoxal phosphate, $3 \cdot 10^{-3} M$ glutamate, $2 \cdot 10^{-4} M$ tetrahydrofolic acid, $1 \cdot 10^{-2} M$

mercaptoethanol and $1.7 \cdot 10^{-2}$ M phosphate, pH 6.8, for 30 min at 36–38°. At the end of the incubation period, 0.2 ml 20% $HClO_4$ was added, followed by 25 μmoles DL-serine as a carrier. The serine was recovered by adsorption on a column of Dowex 50 (H^+ form), oxidized with periodate, and the β-carbon was recovered by precipitation with dimedone (1,1-dimethyl-3,5-diketo-cyclohexane). These procedures, as well as the counting of radioactivity, were carried out essentially as described in the literature[3,6].

The activity of the mitochondrial preparation was almost completely recovered in the disrupted particulate fragments (Table I). The supernatant fraction obtained from the liver homogenate after recovery of the mitochondria, as well as that derived from disrupted mitochondria, were inactive even after fortification with cofactor concentrates. The addition of glutamic acid, tetrahydrofolate, pyridoxal phosphate and mercaptoethanol to the assay mixture was not essential for the activity since their ommission did not decrease significantly the incorporation of ^{14}C into serine. They were added routinely as a precautionary measure because some soluble purified systems showed partial dependence on some of the factors[4]. Glutamate was included in the assay system since transamination to glyoxylate is a possible step in the reaction sequence, and it is known that glutamate is an effective acceptor for the $-NH_2$ group (to yield glutamine[7]).

TABLE I

NET SYNTHESIS OF SERINE FROM GLYCINE

Enzyme	*Volume* ml	*Protein* mg	*Serine* mμmoles	*Serine* mμmoles/g liver (average)
Mitochondria	0.1	1.3	63	
	0.15	2.0	88	820
	0.2	2.7	105	
	0.25	3.4	150	
Sub-mitochondrial particles	0.1	1.0	60	750
	0.2	2.0	99	

The product of the enzyme reaction was identified essentially as described by MITOMA AND GREENBERG[3]. The Dowex-50 column with the adsorbed reaction products was eluted with HCl of increasing concentrations and several fractions were collected. The distribution of radioactivity in the fractions eluted with 0.7 N HCl coincided exactly with the elution profile of serine determined colorimetrically. Two of the main fractions containing serine (24,000 and 47,500 counts/min, respectively) were degraded by periodate oxidation and carbon 3 was obtained as the methylene bismethone. The precipitates from the two samples contained 12,200 and 22,200 counts/min, respectively, or close to one-half of the total activity of serine. This data is consistent with the net synthesis from two molecules of [2-^{14}C]glycine of serine labeled equally with ^{14}C in positions 2 and 3.

Since glyoxylic acid and formaldehyde are suspected intermediates in the conversion of glycine to serine, the effect of addition of these compounds to the reaction mixture was studied. No significant decrease in the amount of ^{14}C incorporated in serine was observed (Table II) which suggests that the intermediates are probably tightly bound to the enzyme complex and are not readily dissociable. In fact, there is ample evidence that the one-carbon fragment from glycine occurs as a derivative of tetrahydrofolic acid[4]. It is of considerable interest that the entire enzyme system, parts of which have been isolated in partially purified form, occurs as an integrated complex firmly attached to mitochondrial structural elements or cristae.

TABLE II

[^{14}C]SERINE SYNTHESIS IN THE PRESENCE OF GLYOXALATE AND FORMALDEHYDE

The amount of protein used was 2.0 mg for mitochondria and 1.6 mg for the sub-mitochondrial particles.

Expt.	*Enzyme*	*Glyoxalate added (µmoles)*	*Formaldehyde added (µmoles)*	*Serine formed (mµmoles)*
1	Mitochondria	—	—	44
		2.5	—	32
		5.0	—	32
2	Mitochondria	—	—	30
		—	5.0	28
		—	10.0	28
3	Particles	—	—	65
		5.0	—	71
		10.0	—	68
4	Particles	—	—	23
		—	5.0	43
		—	10.0	24

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Department of Biochemistry,
University of California Medical School,
San Francisco 22, Calif. (U.S.A.)

D. R. SANADI*
MILDRED J. BENNETT

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* Present address: Gerontology Branch, National institutes of Health, Baltimore City Hospitals, Baltimore 24, Md., U.S.A.

Inhibition of lactic dehydrogenase by α-mercapto-acids

A ternary complex is formed between DPN, lactic acid dehydrogenase and either mercapto-acetate or -succinate[1-3], and thus these mercaptans compete with lactate for the active site on the dehydrogenase[3]. Other thiol compounds tested which are structurally less similar to lactate do not interact either to form complexes with or to inhibit lactic dehydrogenase[3]. To study the relationship of structural similarity of a thiol compound to lactate and its effectiveness as an inhibitor, additional mercapto analogues of lactate, as well as some other sulfur-containing analogues lacking a free -SH group were tested.

Some mercapto analogues were found to be competitive inhibitors of the forward reaction: lactate + DPN ⟶ pyruvate + DPNH, but none inhibited the initial rate of the reverse reaction. This data is compatible with the findings of Van Eys *et al.*[3] that thiol compounds react with DPN and not with DPNH to form ternary complexes with lactic dehydrogenase. Such unidirectional inhibition of the initial rate of the forward reaction by various compounds has been reported by other workers[4,5].

At first glance, it might be thought that such an inhibitory effect could alter the equilibrium constant, but an equation of the kinetics of such an inhibition* shows that the equilibrium constant is independent of the inhibition[6].

The enzyme used in these studies was twice-recrystallized rabbit-muscle lactic dehydrogenase obtained from Sigma Chemical Co. Initial rates of the reactions were

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Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide.

* Kinetic models readily can be devised for this enzyme which show a competitive inhibition of the initial rate, in the forward direction, no inhibition of the initial rate in the reverse direction, and no effect of the inhibitor on the equilibrium constant. Assume, for instance, the following reactions:

$$\begin{tikzcd}
\mathrm{E + DPN} \arrow[d, shift right, harpoon, "K_1"'] & & & \\
\mathrm{E(DPN) + L} \arrow[u, shift right, harpoon] \arrow[r, shift left, harpoon, "K_2"] \arrow[d, shift right, harpoon, "+\mathrm{I}"'] & \mathrm{E(DPN)L} \arrow[l, shift left, harpoon] \arrow[r, shift left, harpoon, "k_f"] & \mathrm{E(DPNH)P} \arrow[l, shift left, harpoon, "k_r"] \arrow[r, shift left, harpoon, "K_3"] & \mathrm{E(DPNH) + P} \arrow[l, shift left, harpoon] \arrow[d, shift right, harpoon] \\
\mathrm{E(DPN)I} \arrow[u, shift right, harpoon, "K_I"'] & & & \mathrm{E + DPNH} \arrow[u, shift right, harpoon, "K_4"']
\end{tikzcd}$$

in which E is enzyme, L is lactate, P is pyruvate, and I is the inhibitor. The enzyme–DPN complex is assumed to combine with either lactate or the inhibitor. All reactions marked with K are assumed to be rapid equilibria, for convenience of calculation. The K's are dissociation constants and the k's are rate constants. Proceeding according to Michaelis and Menten[7], one gets the equation for the rate of reaction, v, in terms of concentration of substrates and total enzyme ($\mathrm{E_T}$).

$$v = \frac{\{k_f K_1 K_2 (\mathrm{DPN})(\mathrm{L}) - k_r K_3 K_4 (\mathrm{DPNH})(\mathrm{P})\}\,(\mathrm{E_T})}{1 + \mathrm{K}_1\,(\mathrm{DPN}) + K_1 K_2 (\mathrm{DPN})(\mathrm{L}) + K_4 (\mathrm{DPNH}) + K_3 K_4 (\mathrm{DPNH})(\mathrm{P}) + K_1 K_I (\mathrm{DPN})(\mathrm{I})}$$

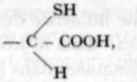
This equation is considerably simplified if one considers initial rates. For the forward reaction (DPNH) and (P) are 0. Then:

$$v = \frac{k_f K_1 K_2 (\mathrm{DPN})(\mathrm{L})(\mathrm{E_T})}{1 + K_1 (\mathrm{DPN}) + \mathrm{K}_1 K_2 (\mathrm{DPN})(\mathrm{L}) + \mathrm{K}_1 K_I (\mathrm{DPN})(\mathrm{I})}$$

which represents a competitive inhibition between I and L, best seen if DPN saturates the enzyme (*i.e.*, the 1 in the denominator drops out). For the reverse reaction, (DPN) and (L) are 0, and so the term with (I) drops out. The reverse reaction is not inhibited by (I). Finally, at equilibrium, $v = 0$. Therefore the numerator must be 0, and $k_f K_1 K_2 (\mathrm{DPN})(\mathrm{L}) = k_r K_3 K_4 (\mathrm{DPNH})(\mathrm{P})$. The equilibrium constant for the overall reaction is (DPNH)(P)/(DPN)(L) and so equals $k_f K_1 K_2 / k_r K_3 K_4$ and is independent of (I).

obtained using a Cary Model 14 *M* spectrophotometer, reading at 340 mμ under first-order conditions with respect to lactate. Additions to the cuvette were made in the following order: various volumes of 0.0166 *M* sodium lactate; 0.3 ml 0.05 *M* DPN; 1.5 ml 0.0166 *M* potassium phosphate buffer, pH 8.6; various volumes of 0.0166 *M* neutralized (pH 8.6) inhibitor solution; enough water to bring the volume to 3.0 ml; and finally 0.2 ml of enzyme solution (prepared by adding 40 mg enzyme/100 ml buffer). Results were plotted according to the method of LINEWEAVER AND BURK[8].

Of the mercapto-analogues of lactate tested during these studies, those which competitively inhibited lactic dehydrogenase were α-mercaptopropionate, α-mercapto-*n*-butyrate, and α-mercapto-*n*-valerate. For the sake of comparison, mercapto-acetate, which had been reported to inhibit lactic dehydrogenase[3,5], was also studied as an inhibitor. The relative degree of inhibition of the compounds at comparable inhibitor and substrate concentration, *i.e.*, at 0.4 ml lactate and 0.3 ml of inhibitor, was 51 % for mercapto-acetate, 61 % for α-mercaptopropionate, 29 % for α-mercapto-*n*-butyrate, and 25 % for α-mercapto-*n*-valerate. This indicates that the degree of inhibition produced by each mercapto-acid is a function of its similarity to lactate. Other evidence of the relationship between the size of the substrate molecule and its affinity for lactic dehydrogenase is that DPNH, in the presence of lactic dehydrogenase, is oxidized more rapidly by pyruvate than by α-keto-*n*-butyrate, and only slowly by α-keto-*n*-valerate[9]. Curiously, although the keto acid analogues of pyruvate react with lactic dehydrogenase to oxidize DPNH, α-hydroxy analogues of lactate, *i.e.*, α-hydroxyacetate, α-hydroxy-*n*-butyrate, and α-hydroxy-*n*-valerate, although tested at relatively high concentrations, did not reduce DPN in the presence of lactic dehydrogenase. Unlike the α-mercapto-acid analogues of lactate, they do not inhibit lactic-dehydrogenase activity in the foreward reaction. Thus the α-mercaptocarboxy group,



$$-\overset{\displaystyle SH}{\underset{\displaystyle H}{C}}-COOH,$$

must have a greater affinity for the reactive site of lactic dehydrogenase than does the α-hydroxycarboxy group.

Evidence that the α-position of the mercapto group in the inhibitors is significant is that β-mercaptopropionic acid did not inhibit lactic dehydrogenase.

Some compounds lacking a free –SH group which were tested as possible inhibitors were thiodiglycol, αα′-thiodipropionic acid, αα′-dithiodipropionic, and phenylmercapto-acetate. None of these inhibited the enzyme. Failure of these compounds to inhibit could be due either to their lack of a free α-SH group or steric hindrance, or possibly a combination of these factors.

The evidence obtained in these inhibition studies lends support to the findings of VAN EYS *et al.*[3] that the interaction of lactic dehydrogenase, DPN, and a mercapto compound is dependent upon the structural similarity of the mercaptan to lactate, and it indicates that the degree of inhibition of lactic dehydrogenase in the forward reaction is a function of the similarity of the thio compound to lactate.

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Department of Zoology, University of California,
Berkeley, Calif. (U.S.A.), and the
University of Redlands, Redlands, Calif. (U.S.A.)

R. R. J. Chaffee
W. L. Bartlett*

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* Present address: University of Southern California Medical School, Los Angeles, Calif. (U.S.A.).

Dosage du phosphate de pyridoxal dans le foie du rat normal, du rat thyrotoxique et du rat thyroidectomisé

Plusieurs auteurs ont constaté une influence des hormones thyroïdiennes sur divers enzymes à phosphate de pyridoxal dans le foie du rat[1–7]: d'une manière générale les activités enzymatiques sont diminuées dans le foie du rat soumis à des injections de thyroxine et sont augmentées chez le rat thyroïdectomisé. L'hypothèse la plus immédiate à la suite de ces résultats est celle d'une action des hormones thyroïdiennes sur le taux de PLP dans le foie, cette hypothèse est d'ailleurs rendue plausible par le fait que, dans certains cas, l'addition *in vitro* de PLP à des préparations de foie de rat thyrotoxique restaure partiellement les activités enzymatiques[3,7]. Dans le présent travail, nous donnons les résultats des dosages de PLP dans le foie du rat mâle normal, du rat mâle thyrotoxique et du rat femelle thyroïdectomisé, animaux chez lesquels des modifications très nettes des décarboxylases de l'acide cystéinesulfinique et de la DOPA[5,6] ainsi que de la cystéinedésulfurase[7] ont été constatées à la suite des traitements reçus. Signalons tout de suite que des dosages de pyridoxal ont également été effectués dans le foie d'animaux normaux et thyroïdectomisés mais que, dans les deux cas, la quantité de ce composé a été trouvé négligeable.

Pour doser le PLP et le pyridoxal, nous avons fait appel à une méthode très voisine de celle décrite par Umbreit *et al.*[8,9] qui utilise l'apotyrosinedécarboxylase

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Abréviations: PLP, phosphate de pyridoxal; DOPA, dihydroxyphénylalanine; ATP, adénosine triphosphate; ACS, acide cystéinesulfinique.

* Nous remercions l'Institut Pasteur qui nous a aimablement fourni cette souche.

existant dans des cellules de *Streptococcus faecalis* souche R (ATCC 8.043)* cultivées sur milieu synthétique privé de vitamine B_6. Le dosage du pyridoxal par activation de l'apotyrosinedécarboxylase est rendu possible grâce au fait que les cellules de *S. faecalis* contiennent aussi une kinase susceptible de catalyser la phosphorylation du pyridoxal en présence de ATP. Soulignons que cette méthode doit cependant être utilisée avec certaines réserves car on peut observer une inhibition de la décarboxylation de la tyrosine chez *S. faecalis* lors d'utilisations de concentrations assez élevées de ATP. Ce phénomène provient vraisemblablement de la formation d'un composé phosphorylé, inhibiteur de la réaction de décarboxylation par compétition avec le phosphate de pyridoxal[10]. Les quelques modifications apportées à la méthode de UMBREIT sont les suivantes: (a) les cultures de *S. faecalis* sont conduites en anaérobiose, ce qui permet d'obtenir des préparations plus actives; (b) la réaction de décarboxylation est suivie à 31° au lieu de 28°; (c) la concentration de ATP utilisée pour le dosage du pyridoxal est de 10 µg par cellule au lieu de 1 mg. Les traitements des animaux, injections de thyroxine et thyroïdectomie, ont été décrits précédemment[5,6]. Les extraits de foie sont préparés de la manière suivante: le foie est broyé dans de l'eau distillée glacée, l'homogénat est amené à un volume égal à 4.5 fois le poids de l'organe. 2 ml d'homogénat, additionnés de 2 ml d'eau distillée et amenés à pH 9 à 10, sont soumis à une hydrolyse alcaline à 100° pendant 10 min selon la méthode de BOXER *et al.*[11]. On filtre sur laine de verre et le filtrat est amené à 200 ml avec de l'eau distillée, les dosages sont effectués sur 0.1 à 1 ml de la solution finale.

Les résultats obtenus sont résumés dans le Tableau I. Les différences de teneur en PLP du foie entre les rats normaux et les rats thyrotoxiques d'une part, les rats normaux et les rats thyroïdectomisés d'autre part, sont statistiquement significatives: diminution de 25 % de la quantité de PLP présent dans le foie à la suite d'injections de thyroxine, augmentation de 40 % après thyroïdectomie.

Si l'on compare nos résultats à ceux de MASCITELLI-CORIANDOLI ET BOLDRINI[12] dont nous avons eu connaissance au cours de notre travail, on peut faire deux remarques. D'une part, les chiffres obtenus par ces auteurs chez l'animal normal (1.55 ± 0.08 µg de PLP/g de foie frais) sont beaucoup plus faibles que ceux présentés ici (5 µg PLP/g de foie frais, calculés d'après nos résultats en admettant que 1 g de foie

TABLEAU I

DOSAGES DE PHOSPHATE DE PYRIDOXAL DANS LE FOIE DU RAT NORMAL,
DU RAT THYROTOXIQUE ET DU RAT THYROÏDECTOMISÉ

Les injections quotidiennes de 500 µg de DL-thyroxine sont effectuées pendant 12 jours avant les dosages (Exp. I); la thyroidectomie a lieu trois semaines avant les dosages (Exp. II). Les animaux utilisés pèsent de 100 à 110 g avant les traitements.

	Exp. I		*Exp. II*	
	Témoins	*Injectés*	*Témoins*	*Thyroidectomisés*
Nombre de rats	7	7	8	8
Poids des foies	5.50 ± 0.3*	5.20 ± 0.35	6.90 ± 0.30	3.30 ± 0.20
Quantité de phosphate de pyridoxal (sel de Na) (mµg/mg poids sec)	25.5 ± 2.0	19.0 ± 1.5	26.5 ± 2.0	36.5 ± 2.5
		0.01 < P < 0.02**		P = 0.01

* Déviation standard de la moyenne.

** P = facteur de probabilité, par rapport aux animaux témoins.

frais correspond à environ 200 mg de poids sec). Ces différences proviennent peut-être du fait que les rats sont soumis à des régimes alimentaires différents, régimes plus ou moins riches en vitamine B_6 ou modifiant plus ou moins la quantité de coenzyme existant dans le foie. Nos rats reçoivent l'alimentation habituelle de l'élevage, c'est-à-dire un régime non synthétique (biscuits "Causse") alors que les rats de **Mascitelli-Coriandoli** et **Boldrini** sont soumis à un régime synthétique. Or, nous avions remarqué au cours d'expériences antérieures[13] que certains régimes synthétiques provoquent une diminution de la décarboxylase de l'acide cystéinesulfinique dont le niveau d'activité est particulièrement dépendant de la quantité de PLP présente dans les tissus. D'autre part, la diminution, observée par **Mascitelli-Coriandoli et Boldrini**, de la quantité de PLP chez l'animal traité par la thyroxine (diminution de 50 à 60 %) est nettement plus forte que celle obtenue ici, et ceci bien que la quantité de thyroxine injectée par ces auteurs soit de l'ordre de 10 à 20 μg par animal et par jour, doses qui ne provoquent dans nos expériences, chez des rats soumis à un régime non synthétique, aucune modification d'activité de plusieurs enzymes à phosphate de pyridoxal[5,7]. Une hypothèse serait que des rats ayant au départ une teneur plus faible de PLP dans le foie sont plus sensibles à la thyroxine; toutefois, il se pourrait que cette sensibilité particulière soit due non pas à ce seul facteur mais à l'ensemble des conditions de traitement, en particulier au régime, auxquelles sont soumis les animaux.

Un autre fait retient l'attention: dans nos expériences, les diminutions et augmentations de la quantité de PLP présent dans le foie à la suite d'injections de thyroxine ou de thyroïdectomie paraissent relativement faibles en comparaison des diminutions et augmentations de certaines activités enzymatiques constatées après les mêmes traitements (la cystéinedésulfurase diminue de 50 %, la décarboxylase de ACS de 70 % après des injections de thyroxine), de sorte qu'il est permis de se demander si seule la variation du taux de coenzyme peut rendre compte des effets produits sur les enzymes à phosphate de pyridoxal par les hormones thyroïdiennes.

Ces expériences ont été réalisées avec la participation technique de C. **Portemer** que nous remercions de son efficacité.

Laboratoire de Chimie biologique, Faculté des Sciences,
Paris (France)

J. **Labouesse**
F. **Chatagner**
B. **Jollès-Bergeret**

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Detection of the cerebrosides on paper chromatograms

The cerebrosides are unusually resistant to many colorimetric reactions designed for paper chromatograms because they are almost insoluble in many solvents, and their galactoside and amide bonds are stable under the mild reaction conditions generally used with paper chromatograms.

The identification of cerebrosides and other lipids with iodine was reported recently[1]. This is a somewhat general test, probably depending upon the presence of the double bond in sphingosine. Two more specific reactions are described below.

1. The galactoside bond can be hydrolysed with trichloroacetic acid in chloroform. (Dissolve 20 g of the acid in 5 ml water and dilute to 100 ml with chloroform.) The chromatogram is sprayed heavily, placed in a cool-draft oven and warmed to 80°. The paper is heated until trichloroacetic acid is no longer detectable by smell (about 40 min). It is then sprayed with aniline-oxalate reagent[2] and heated at 100° for 5 min. The compounds appear as fluorescent spots under u.v. light. Before the hydrolysis, it is advisable to apply test spots of cerebrosides to unused portions of the paper. These are also sprayed with aniline-oxalate reagent to make certain that the compounds have reacted with trichloroacetic acid. The hydrolysis step can be repeated if necessary. As little as 25 μg phrenosine in a 2 cm^2 area can be detected by this method.

2. The active hydrogen of the secondary amide bond in the cerebroside molecule can be replaced by halogens. Reaction with chlorine gas[3] is difficult to control and aq. NaOCl is nonreactive with these water-insoluble compounds. A chloroform solution of chlorine is prepared by the successive addition of 5 ml 5 % NaOCl and 5 ml glacial acetic acid to 100 ml chloroform[4]. The green chloroform solution of chlorine is sprayed heavily on the chromatogram. The paper is dried in air, sprayed with 95 % ethanol, redried, then sprayed with benzidine–KI reagent (22 mg KI and 2 g benzidine–HCl dissolved in 200 ml 50 %, v/v, ethanol)[5]. The benzidine is oxidized at the cerebroside loci to give blue spots on a white background. The reaction is very marked with 10 μg phrenosine/2 cm^2.

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Department of Biochemistry, The School of Medicine, ANN E. BRESLER*
University of Pennsylvania, Philadelphia, Penn. (*U.S.A.*)

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* Present address: Department of Microbiology, New York University College of Medicine, NewYork, New York.

Preliminary Notes

Thymidine diphosphate mannose from *Streptomyces griseus*

During an investigation of the nucleotides of *Streptomyces griseus* two thymidine derivatives have been isolated. Evidence is presented here which indicates that one of these is a new nucleotide, thymidine diphosphate mannose, and the other is thymidine diphosphate rhamnose, the existence of which was reported recently[1] in *Lactobacillus acidophilus* R-26 and *Escherichia coli* 15 T^-.

An alcoholic extract of *S. griseus* mycelium (kindly supplied by Glaxo Laboratories, Ltd.) which contained the soluble nucleotides of the mould was subjected to chromatography on a column of Dowex-1 (X2) resin (chloride form), elution being effected by linear gradient[2] with LiCl. The fraction containing the two thymidine derivatives was eluted between uridine diphosphate glucose and uridine diphosphate. After neutralisation with LiOH and evaporation, LiCl was removed by solution in an acetone–alcohol mixture. Further purification was effected by chromatography on Whatman No. 3 paper in ethanol–ammonium acetate[3], pH 3.8.

The mixture was hydrolysed at pH 1 for 15 min at 100° and the products examined by paper chromatography in butanol–pyridine–water[4] and phenol–water[5] with the alkaline $AgNO_3$[6] and phloroglucinol–HCl[7] reagents. Two sugars with the R_{ribose} values of mannose and rhamnose were detected, and their presence was confirmed by electrophoresis on a borate-buffered (pH 9.2) paper[8]. The sugars were further characterized by reduction[9] with $NaBH_4$ to the corresponding alcohols. These were identified by partial conversion to anhydro-polyols through the action of mineral acid[10]. The pattern, colour and relative intensities of the spots corresponding to anhydro-polyols were identical to that given by a mixture of mannitol and rhamnitol (4:1) which had been treated similarly.

When the nucleotides were hydrolysed for 3 h at 100° in 6 *N* HCl the only base formed was thymine, identified chromatographically (2 solvents) and from its absorption spectrum. Moreover, hydrolysis at pH 1 for 15 min at 100°, followed by treatment with *Crotalus adamanteus* venom at pH 9, liberated only one nucleoside with the same R_F as thymidine in two solvents. It gave a positive reaction with the cysteine–H_2SO_4 (ref. 11) and a negative reaction with the periodate–Schiff[12, 13] reagents. The ratio thymidine: total phosphate[14]: labile phosphate: sugar[15] was 1.04: 2.0: 0.96: 1.06 for the mixture of the two nucleotides.

These observations indicate the presence in this organism of two nucleotides in which thymidine is joined at the 5′-position through a pyrophosphate residue to either mannose or rhamnose. The ready liberation of thymidine by the action of *Crotalus adamanteus* venom indicates that the pyrophosphate is at the 5′-position, since it is known[16] that this venom contains a powerful 5′-nucleotidase but has little action on nucleoside 3′-phosphates. The absence of reducing properties before hydrolysis supports the normal structure for this class of nucleotides in which the pyrophosphate is attached to the 1-position of the hexose.

Thymidine derivatives of this type are uncommon and only recently have thymidine diphosphate rhamnose[1] and related deoxysugar nucleotides[17] been described. Guanosine diphosphate mannose[18] is the only other nucleotide containing mannose. Thymidine diphosphate mannose is of interest as a possible intermediate in the biosynthesis of streptomycin B (α-D-mannopyranosyl-streptomycin)[19], which occurs together with streptomycin in this micro-organism.

Department of Chemistry, King's College, University of Durham,
Newcastle upon Tyne (Great Britain)
J. BADDILEY
N. L. BLUMSOM

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The isolation of guanosine diphosphate colitose from *Escherichia coli*

The specific immunochemical reactions exhibited by bacterial endotoxins (lipopolysaccharides) are primarily determined by the 3,6-dideoxyhexose components of these substances[1]. LUDERITZ *et al.*[2] reported that colitose (3,6-dideoxy-L-galactose) is the dideoxyhexose of a lipopolysaccharide isolated from *Escherichia coli* 0111-B_4. A colitose-containing nucleotide, guanosine diphosphate colitose, has now been isolated from this organism*.

Cells were grown in a continuous-culture apparatus, harvested during exponential growth and washed with water. 1 kg (wet weight) of cells was extracted with cold 10 % trichloroacetic acid, the precipitate removed by centrifugation and the supernatant fluid extracted 5 times with ice-cold ether. The ultraviolet-absorbing material in the neutral extract was fractionated on Dowex-1 (Cl^- form) resin using LiCl as

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* A culture of *E. coli* 0111-B_4 was kindly supplied by Dr. Pearl L. Kendrick, School of Public Health, University of Michigan, Ann Arbor, Mich.

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the eluting agent. The crude nucleotide fraction, eluted with 0.25 M to 0.35 M LiCl, was concentrated and the lithium salts of the nucleotides were precipitated. Analysis of a portion of this fraction, after hydrolysis at 100° in 0.01 N HCl for 10 min, indicated the presence of colitose as determined by paper chromatography and the reaction obtained with 2-thiobarbituric acid[*]. No other reducing substances were detected on the chromatograms with the ammoniacal $AgNO_3$ reagent[4]. Colitose was not detected in the crude nucleotide fraction prior to acid hydrolysis. Further fractionation of the crude lithium salts by treatment with charcoal, paper chromatography and paper ionophoresis yielded 2.5 μmoles of a colitose-containing nucleotide. Paper chromatography in two solvent systems and ionophoresis[**] indicated that the substance was homogeneous; in each case, hydrolysis of the ultraviolet-absorbing spot yielded colitose. The ultraviolet-absorption spectrum of the nucleotide was identical with that of guanosine monophosphate at pH's 1.0, 7.0 and 11.0. Analysis of the material indicated the following molar ratios: guanine, 1.0; organic phosphorus, 1.97; colitose, 0.95. The colitose moiety was further characterized, following hydrolysis of the nucleotide, by comparison with authentic colitose[***] in three paper-chromatographic solvent systems. Similarly, the only ultraviolet-absorbing material present in such an hydrolysate was chromatographically indistinguishable from guanosine diphosphate[5]. The nucleotide exhibited a negative reaction with diphenylamine although deoxyguanosine monophosphate gave a typical blue color under the same conditions. The nucleotide apparently is guanosine diphosphate colitose.

The occurrence of a guanosine nucleotide containing colitose suggests a possible metabolic relationship to the other known guanosine sugar nucleotides such as guanosine diphosphate mannose and guanosine diphosphate fucose; the relationship between the latter compounds already has been demonstrated[6].

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Rackham Arthritis Research Unit and Department of Bacteriology, EDWARD C. HEATH
The University of Michigan, Ann Arbor, Mich. (*U.S.A.*)

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[*] Oxidation of colitose with periodic acid yields malonyl dialdehyde which reacts with 2-thiobarbituric acid to give a pink-colored complex absorbing maximally at 532 mμ (ref. 3).

[**] Chromatography of sugars was carried out on Whatman No. 1 paper in the following solvent systems: (*a*) butanol (6)–pyridine (4)–water (3); (*b*) ethyl acetate (3)–acetic acid (1)–water (3); (*c*) butanol (10)–ethanol (1)–water (2). Paper ionophoresis was carried out on Whatman No. 3MM paper at 10 V/cm in 0.05 M phosphate buffer, pH 7.4.

[***] We are grateful to Dr. A. M. Staub, L'Institut Pasteur, Paris, for immunochemically authenticating our preparation of colitose. A sample of synthetic colitose was kindly supplied by Dr. O. Westphal, Dr. A. Wander Forschungsinstitut, Freiburg-Zähringen, Germany.

Oxydation de la ribonucléase et de l' α-lactalbumine par la polyphénoloxydase de champignon

L'étude manométrique et spectrophotométrique de l'oxydationde nombreuses protéines par la polyphénoloxydase[1-8] indique que cette oxydation porte sur les restes de tyrosine de la chaine polypeptidique. Certains de ces travaux[1,3] prêtent à des critiques[2] portant sur la pureté des protéines-substrats et sur celle de l'enzyme utilisée ainsi que sur les conditions de l'incubation. La DOPA-quinone paraît être le produit intermédiaire vraisemblable de l'oxydation des restes de tyrosine de nombreux peptides[9]. Il importait cependant de connaître sur le plan chimique la réalité de la disparition de la tyrosine et la nature des produits formés par son oxydation. La DOPA-quinone devant être considérée comme un des premiers termes de l'oxydation de la tyrosine par la polyphénoloxydase, on pouvait penser que, au sein des protéines, sa stabilisation par les liaisons peptidiques, permettrait de la caractériser sous forme de restes de DOPA en utilisant des conditions d'incubation telles que les restes de DOPA-quinone seraient réduits au fur et à mesure de leur formation, ce que les expériences décrites ici ont montré.

Celles-ci ont été réalisées avec des protéines homogènes (ribonucléase et α-lactalbumine) et une polyphénoloxydase de champignon très pure de haute activité spécifique[10].

La consommation d'oxygène, la disparition de tyrosine, la caractérisation ou le dosage de la DOPA et l'activité enzymatique pour la RNase ont été étudiées après incubation des protéines en présence de polyphénoloxydase.

Les résultats obtenus sont réunis dans le Tableau I. Ils montrent que la RNase n'est pas oxydée par la polyphénoloxydase en présence ou en l'absence de catéchol, l'activité étant partiellement ou totalement conservée. Sous forme oxydée par l'acide performique[11], les 6 restes de tyrosine que contient la molécule sont oxydés sans que la DOPA puisse être caractérisée. Par contre, l'incubation en présence d'acide ascorbique en excès donne une oxydation des 6 restes de tyrosine dont 3 ont pu être retrouvés sous forme de DOPA après hydrolyse.

L'α-lactalbumine native est oxydée et 3 restes de tyrosine sur les 5 présents ont disparu. La DOPA a également été caractérisée après oxydation en présence d'acide ascorbique.

Ces faits montrent que:

(1) L'oxydation de la RNase et de l'α-lactalbumine par la polyphénoloxydase intéresse bien les restes de tyrosine (pour chaque mole d'O_2 consommé, il y a disparition d'un reste de tyrosine).

(2) La caractérisation et le dosage de la DOPA après hydrolyse des protéines oxydées en présence d'acide ascorbique impliquent la transformation intermédiaire des restes de tyrosine en restes de DOPA-quinone.

(3) Le caractère de non-oxydabilité de la RNase native est lié à l'existence de la structure secondaire de cette protéine.

(4) L'oxydation de la RNase performique par la polyphénoloxydase a permis d'obtenir pour la première fois un dérivé d'une chaîne polypeptidique naturelle où tous les restes de tyrosine ont été transformés en restes de DOPA. Ses propriétés

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Abréviations: DOPA, dihydroxyphénylalanine; RNase, ribonucléase; RNase-performique, RNase oxydée par l'acide performique.

TABLEAU I

Composition des essais[a]			*Resultats*		
Protéines étudiées	*Substances ajoutées au milieu*	*Temps incubation (min)*	*O_2 consommé (en moles/mole de protéine oxydée)*	*Tyrosine disparue (en restes/mole de protéine oxydée)*[b]	*DOPA apparue (en restes/mole de protéine oxydée)*[c]
Ribonucléase native[d, e]		360	0	0	0
Ribonucléase native	catéchol	210	0	0	0
Ribonucléase native	catéchol et (ou) acide ascorbique	30 à 300	0	0	0
Ribonucléase oxydée par l'acide performique	—	60	5.8	5.9	
Ribonucléase oxydée par l'acide performique	catéchol	420	5.6[f]	5.9	—
Ribonucléase oxydée par l'acide performique	acide ascorbique[h]	22	—	5.8	3
α-Lactalbumine[g]	—	90	3	3	0
α-Lactalbumine[g]	acide ascorbique[h]	36	—	2.7	1.5

[a] Incubations en tampon phosphate 0.1 *M*, pH = 6.8; 100 agitations par min en appareil de Warburg conventionnel. Les milieux contenaient de 6–12 mg de protéines et 0.040–0.060 ml d'enzyme (1 ml contient 13 mg de protéine et 57,500 U, ref. 12).

[b] Dosée après hydrolyse (HCl 6 *N*, 24 h) par réaction à l'α-nitroso-β-naphtol[13] et par spectrophotométrie après séparation sur Dowex 50.

[c] Hydrolyse par HCOOH 5 *N* en présence de Dowex 50. Dosage colorimétrique en utilisant la réaction d'Arnow[14] modifiée et spectrophotométrique après séparation sur Dowex 50. Cette méthode a permis, au cours des essais, de doser 50% environ de la DOPA libre ajoutée au milieu avant hydrolyse (techniques originales en cours de publication).

[d] 3 × recristallisée (Worthington) $E^{1\%}_{1\,\mathrm{cm}}$ 2775 Å = 6.8.

[e] En outre, l'activité de la ribonucléase[15] a été étudiée après incubation. Les résultats ont montré qu'en l'absence de catéchol il n'y avait pas de perte d'activité mais qu'en sa présence on perdait 88% de celle-ci recouvrable partiellement après addition d'acide ascorbique.

[f] Différence avec le témoin catéchol seul en présence de polyphénoloxyden.

[g] 3 fois recristallisée $E^{1\%}_{1\,\mathrm{cm}}$ 2800 Å = 20.9.

[h] 30 moles par reste de tyrosine présent dans le substrat.

chromatographiques, spectrophotométriques, électrophorétiques et de comportement à l'hydrolyse ont été étudiées. Elles seront décrites ultérieurement.

Nous remercions le Professeur Ottesen (Copenhague) pour le don généreux d'α-lactalbumine et le Professeur Kertesz (Tunis) pour celui de la polyphénoloxydase utilisée, ainsi que pour ses utiles suggestions et critiques au cours de ce travail.

Laboratoire de Chimie Biologique, Faculté de Médecine,
Marseille (France)

S. Lissitzky
M. Rolland
S. Lasry

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Sulfur-containing deoxypentose polynucleotides obtained from *Chlorella* cells

The role of sulfur in the cell division of *Chlorella* has been studied in our laboratory using the technique of synchronous culture[1-6]. It was demonstrated that, in a medium deficient in sulfur, the cells of the alga *Chlorella ellipsoidea* were unable to divide, although the cell mass and nuclear substances increased to some extent. When transferred to a medium containing sulfate only, these sulfur-deficient cells became able to perform cell division under non-photosynthesizing conditions, but not under photosynthesizing conditions, indicating that there exist some antagonistic relations between the anabolic (photosynthetic) metabolism and the formative metabolism leading to the process of cell division. To make the sulfur-starved cells capable of cell division under photosynthesizing conditions, a source of nitrogen (nitrate), in addition to sulfur (sulfate), had to be supplied in the suspending medium.

Using ^{35}S as a tracer the fate of sulfur supplied to the sulfur-deficient cells under non-photosynthesizing and photosynthesizing conditions was investigated. The cell material was fractionated by the method of SCHMIDT–THANNHAUSER–SCHNEIDER[7] or by that of OGUR AND ROSEN[8] (see also ref. 9). It was found that when the sulfur-starved cells were supplied with sulfate under non-photosynthesizing conditions, the sulfur went, in the largest amount, into the fraction soluble in hot acid (DNA fraction) during the process of cell division. When the sulfur-starved cells were induced to cell division by being supplied with sulfate and nitrate under photosynthesizing conditions, most of the sulfur went into the protein fraction, and comparatively little was found in the DNA fraction.

As far as the electrophoretic pattern (followed by ^{35}S radioactivity) was concerned,

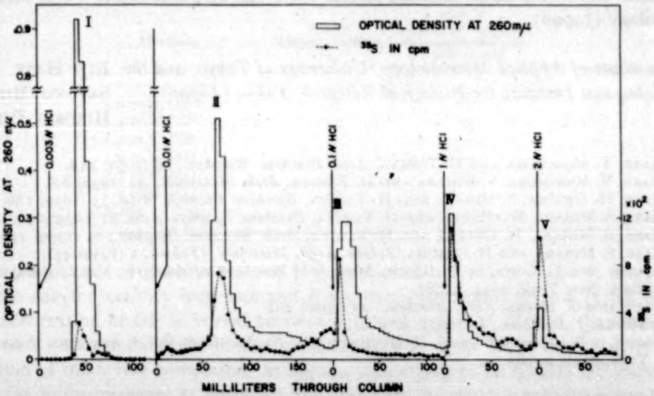

Fig. 1. Anion-exchange chromatogram of the DNA fraction obtained from synchronously grown *Chlorella* cells at the completion of division. Cells, which had been made incapable of cell division by the deficiency of sulfur, were supplied with $^{35}SO_4^{=}$ and nitrate to provoke their division under photosynthesizing conditions. The DNA fraction was isolated by the method of SCHMIDT-THANNHAUSER–SCHNEIDER[7]. Exchanger: Dowex-1-chloride, 200–400 mesh, 10 cm × 1 cm².

Abbreviation: DNA, deoxypentosenucleic acid.

the DNA fraction obtained at the completion of cell division appeared to contain a definite group of sulfur substances, irrespective as to whether the division took place under photosynthesizing or non-photosynthesizing conditions. As may be seen from an anion-exchange chromatogram reproduced in Fig. 1, these sulfur substances were more or less intimately associated with u.v.-absorbing substances. When tested with cysteine and 75 % H_2SO_4 (ref. 10), Fractions I, II and III gave pink colors, while Fractions IV and V gave apparently negative reactions. Paper chromatographic analyses of each fraction thus separated revealed that the majority of the radioactive spots, except those obtained from Fraction III, showed a distinct u.v. absorption. The u.v.-absorbing substances contained in Fraction I were, however, found to be mainly free purine bases having, by themselves, no radioactivity. The radioactivity found in this fraction seemed, therefore, to be due to a small amount of contaminating purine nucleosides. It seems likely that, during the extracting process using hot acid, the sulfur, which had originally been bound to the sugar moiety of purine nucleotides, was split from the bases in the form of sulfur-containing deoxypentose (with or without phosphate), and that the sulfur-containing moiety thus split might have appeared in Fraction III. This inference was also supported by electrophoretic experiments. In an electrophoresis at pH 5, a part of the ^{35}S in the DNA fraction moved rapidly toward the anode without being accompanied by u.v.-absorbing substances, while the rest of the ^{35}S was distributed in the two major peaks with less mobility, accompanied by u.v.-absorbing substances.

Experiments carried out to date to clarify the nature of the sulfur-containing moiety described above showed that it was neither cyst(e)ine nor methionine. At any rate, the results described above give us ground for the belief that the sulfur in the DNA fraction is in some form bound either to DNA or to some other deoxypentose polynucleotides. A detailed account of this work will be published in Plant and Cell Physiology (Japan).

The Institute of Applied Microbiology, University of Tokyo, and the Tokugawa Institute for Biological Research, Tokyo (Japan)

EIJI HASE
SAYOKO MIHARA
HIROSHI TAMIYA

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The effect of metal-binding agents on aldehyde dehydrogenases

Baker's yeast contains two aldehyde dehydrogenases, the DPN-linked[1,2], activated by K+, and the TPN-specific[3,4] activated by divalent cations. It is well known that thiol compounds or other metal-binding substances are required for the activity of the former enzyme[1,2,5] although no explanation of the activation effect can be given at present[5]. Metal-binding agents react with zinc metallodehydrogenases[6]. According to the experimental conditions, OP is either a competitive inhibitor with respect to DPN[6] or an enzyme activator[7], which indicates that zinc atoms are involved in the interaction of the coenzyme with the catalytic protein.

The effect of OP and HQ on the DPN-specific aldehyde dehydrogenase of yeast is affected by cysteine, which is added to the reaction mixture in order to obtain full enzyme activity. With cysteine, these substances are "instantaneous" reversible inhibitors (Table I, Expt. A) the degree of inhibition produced by a given concentration of complexing agent being a function of the concentration of DPN. Variation of acetaldehyde concentration does not affect the action of the inhibitor. Plots of reciprocal enzyme activity *versus* reciprocal DPN concentration, at several concentrations of inhibitor, give straight lines with a common intercept, as in competitive inhibition, and from these plots the effect of the inhibitor on K_{DPN} can be calculated. Both OP and HQ compete with DPN and increase the value of K_{DPN}.

TABLE I

EFFECT OF OP AND HQ ON DPN-LINKED ALDEHYDE DEHYDROGENASE OF YEAST

The reaction mixture contained 0.1 *M* Tris buffer, pH 8.0; 1 m*M* cysteine (Expt. A only); 0.44 m*M* DPN; 0.05 *M* KCl, $1.7 \cdot 10^{-4}$ *M* acetaldehyde and 17 µg of enzyme preparation[5]. Final volume, 3.0 ml. Enzyme activity measured at 21° by the increase (per min) in absorbancy of the reaction mixture at 340 mµ, after the addition of acetaldehyde.

Additions	*Expt. A* *Enzyme inhibition* *(%)*	*Expt. B* *Activity in relation to the enzyme treated with 1 mM cysteine* *(%)*
5.0 m*M* OP	48	29
2.4 m*M* OP	25	53
1.2 m*M* OP	19	58
5.0 m*M* HQ	87	—
2.5 m*M* HQ	45	62
1.0 m*M* HQ	10	—
None	—	6

In the absence of cysteine, OP and HQ are enzyme activators (Table I, Expt. B). Thus the enzyme activity increases and K_{DPN} decreases from 1.46 to 0.33 mM when the concentration of OP is varied between 0.33 and 1.30 m*M*, that is, the activator promotes a closer association of the catalytic protein with DPN and thereby the formation of the active compound. At higher concentrations (≥ 5 m*M*) OP increases K_{DPN} (as in the presence of cysteine) and therefore the enzyme activity is less than that corresponding to the optimal concentration of activator (1.30 m*M*).

When the enzyme and OP are incubated before the activity is measured, a

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Abbreviations: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; OP, 1,10-phenanthroline; HQ, 8-hydroxyquinoline; EDTA, ethylenediaminetetraacetate; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; BAL, 2,3-dimercaptopropanol.

time-dependent, irreversible inhibition results. This can be observed also with BAL but not with the same or higher concentrations of HQ, zincon, diethyldithiocarbamate, thiourea, cupferron, sodium azide, 4,4′-dipyridyl, histidine, glycine, EDTA, cyanide or cysteine. Nevertheless, cysteine, HQ, cyanide and EDTA prevent completely the irreversible action of OP (Table II) and with cysteine there is a close relationship between the effect on the enzyme activity, and on the inactivation with OP. The protection effect indicates that a metal is the common site of action of the enzyme activators and, according to the mechanism of inhibition (and activation) with OP, the metal atom should be a point of attachment of DPN to the protein. These results fit in well with the scheme proposed by VAN EYS *et al.*[7] for zinc metallodehydrogenases.

TABLE II

EFFECT OF ACTIVATORS OF DPN ALDEHYDE DEHYDROGENASE OF YEAST ON THE ENZYME IRREVERSIBLE INACTIVATION BY OP

0.25 mg (Expt. A), 0.15 mg (Expt. B) or 0.36 mg (Expt. C) of enzyme preparation in 0.5 ml 0.1 *M* Tris buffer, pH 8.0, with 0.05 *M* KCl and 0.3 m*M* OP was treated with additions as shown for 66 min (Expt. A) or 55 min (Expts. B and C). Enzyme activity measured in 0.02 ml of these extracts as described in Table I (Expt. A). The figures include the respective controls.

Expt.	*Additions*	*Inactivation* (%)	*Protection* (%)
A	None	69	—
A	1 m*M* cysteine	0	100
B	None	100	—
B	1 m*M* EDTA	0	100
B	1 m*M* KCN	0	100
C	None	62	—
C	0.3 m*M* HQ	0	100

Similar studies are in progress with the TPN-specific aldehyde dehydrogenase of yeast and liver aldehyde dehydrogenase[8]. Although these enzymes do not require an organic activator[5], they are sensitive to metal-binding agents. Thus, the Seegmiller enzyme is inhibited 72 % by OP (0.5 m*M*), 83 % by diethyldithiocarbamate (0.5 m*M*), and 31 % by cupferron (0.5 m*M*). Also the liver aldehyde dehydrogenase is inhibited 47 % by OP (1 m*M*), 73 % by HQ (1 m*M*) and 78 % by diethyldithiocarbamate (1 m*M*).

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Institute of Biochemistry, School of Medicine,
University of Buenos Aires (Argentina)
M. N. SCHWARCZ
A. O. M. STOPPANI

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A METHOD FOR THE DETERMINATION OF STABILITY CONSTANTS OF SOME ENZYME-METAL COMPLEXES

MARK M. JONES

Department of Chemistry, Vanderbilt University, Nashville, Tenn. (U.S.A.)

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SUMMARY

A method is presented which can be used to determine the stability constants of moderately stable enzyme–metal complexes. The data required are those giving the relationship between metal ion concentration and enzyme activity over a range of metal ion concentrations but at constant concentration of substrate. Where the metal is complexed to independent groups, the equilibrium constant is obtained by determing the active enzyme: inactive enzyme ratio and estimating the free metal ion concentration. This method may be used even with very impure enzyme preparations if the ratio active enzyme: inactive enzyme changes slowly enough with changes in the metal ion concentration. The application of this method to the L-leucine aminoexopeptidase–manganous ion complex gives a value of $\log_{10}$ of the stability constant of 4.65 at 40° and an ionic strength of 0.1 and a pH = 8.

INTRODUCTION

The determination of the equilibrium constants for the reactions of enzymes with metal ions (for both activation and inactivation processes) is of importance as a quantitative estimate of the stability of the complex formed. In any such procedure it is necessary to use a model for the complex if an equilibrium constant is to be evaluated. Unfortunately, a really accurate model is difficult to devise in the absence of detailed information on the structure of the enzyme. The principal assumption made in the following treatment is dictated by the complexity of the coordinating agent; it is simply that the coordination positions of the enzyme function independently of one another.

This method is essentially a generalization of one presented by MARTELL AND CALVIN[1] in 1952. This was an approximate method based upon the effect of the metal in activating the enzyme. They applied this method to the data of SMITH[2] on the effect of manganous ion on the activity of L-leucine aminoexopeptidase. In brief, the activating equilibrium is represented as

$$\text{protein} + \text{Mn}^{2+} \rightleftharpoons \text{enzyme}$$

and for this equilibrium they wrote the equilibrium constant (essentially the stability constant of the enzyme) as

$$K = \frac{[\text{enzyme}]}{[\text{protein}]\,[\text{Mn}^{2+}]}$$

where [enzyme] and [protein] refer to the concentration of coordination positions on the enzyme occupied by metal ions and the concentration of donor positions which can still combine with the metal, respectively. The concentration of manganous ion in solution is written $[Mn^{2+}]$. At the point where the enzyme has 50 % of its limiting activity, [protein] = [enzyme] and $K = 1/[Mn^{2+}]$, the concentration of free manganese in the system.

The most serious objections to the above procedure are (a) that it does not take into consideration any change in the concentration of metal ion due to complexation to form the enzyme, nor does it suggest how this assumption might be verified; and (b) that it can only be used where an estimate of the limiting proteolytic coefficient can be estimated visually from a graph of proteolytic coefficient *vs.* $-\log [Mn^{2+}]$. While the first objection can be obviated by a direct experimental determination of the free metal ion concentration, most such determinations are of low relative precision. The change in behavior of the enzyme is at least as sensitive an indicator of the free metal ion concentration as most available methods. This is particularly evident when the wide range of metal ion concentration used is considered. The following procedure can be applied to data such as SMITH's in such a manner as very drastically to reduce or completely eliminate the error which such an assumption can introduce into the stability constant calculation. The second objection can be eliminated by a simple extrapolation procedure explained below.

The steps in this method can be illustrated by a consideration of the data of SMITH (loc. cit.). This data is a sigmoid curve of relative enzyme activity *vs.* $-\log_{10} [Mn^{2+}]$ at constant substrate concentration. From data of this type it is possible to obtain the limiting enzymic activity by graphical means. This is done by using a plot of $1/C_{LA}$ *vs.* $1/[Mn^{2+}]$ following the procedure of LINEWEAVER AND BURK[3]. (C_{LA} is the

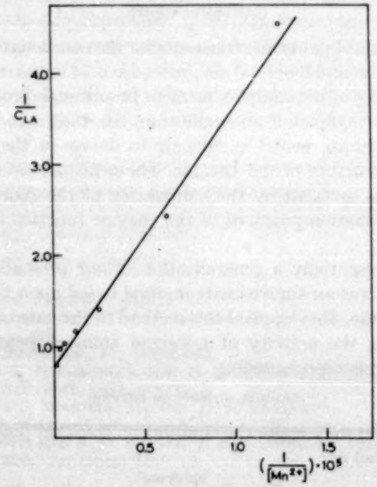

Fig. 1. Graphical extrapolation to obtain the limiting value of the proteolytic coefficient.

proteolytic coefficient for L-leucine aminoexopeptidase and is equal to K'/E where K' is the first order velocity constant at enzyme concentration E). This is simply a convenient method for the treatment of such sigmoid curves for purposes of extrapolation. Alternatively the procedure of DIXON[4] can be used for this purpose. This treatment of SMITH's data is shown in Fig. 1. This gives a limiting value of C_{LA} at enormous concentrations of manganous ion as 1.30. This procedure can also be used to determine the limiting value of the proteolytic coefficient when the range of metal ion concentration is so small that the procedure of MARTELL AND CALVIN[1] cannot be used. Their procedure requires that this limiting value be nearly attained in one or more of the experimental values; this procedure has no such requirement. Once this limiting value is established it can be used to determine the ratio enzyme: protein using $C_{LA}/(C_{LA})_\infty - C_{LA}) = [\text{enzyme}]/[\text{protein}]$ where $(C_{LA})_\infty$ is the limiting value. The equilibrium constants are obtained simply by dividing this factor by the concentration of manganous ion. These results are collected in Table I. Graphical extrapolation gives $-\mathrm{p}K = 4.65$ for this formation constant as the concentration of manganous ion approaches zero. This extrapolation to zero metal ion concentration is justified by the fact that very large concentrations of manganous ion slowly inactivate the enzyme, presumably by hydrolysis. Another reason for such a procedure is that a small error in the ratio $C_{LA}/(C_{LA})_\infty$ causes a large error in the stability constant when this ratio is close to unity. With other metal–enzyme systems a significant decrease in K may be found to occur as the metal ion concentration decreases. This indicates that the free metal ion concentration is much less than the total metal ion concentration. In such a case the K's calculated for these metal ion concentrations should be ignored.

TABLE I

STABILITY CONSTANTS FOR THE L-LEUCINE AMINOEXOPEPTIDASE MANGANOUS ION COMPLEX AT 40°

$[Mn^{2+}] \cdot 10^5$	C_{LA}	K	$-pK$
800	1.26	3,670	3.564
80	1.18	12,350	4.091
32	1.02	11,800	4.072
16	0.97	18,400	4.264
8.0	0.86	24.400	4.386
4.0	0.71	31,300	4.941
1.60	0.41	28,800	4.458
0.80	0.22	25.400	4.404
0.32	0.15	43.500	4.638

The rather considerable range of the K values illustrates the difficulties associated with the determination of such constants. The range could probably be somewhat reduced by running the reaction in a medium of constant ionic strength while varying the concentration of the individual species[5,6]. This would be attended by some difficulties as the enzymic process generates weak electrolyte and it would also possibly be adversely effected by the very high electrolyte concentrations generally used for this purpose (*e.g.* $\sim 3\,M$ in a uni-univalent electrolyte). Attempts to correct the K values for the activity coefficients are difficult to carry out on the present measurements, partly because of the high ionic strength and partly because of the difficulty of estimating activity coefficients for the coordination sites on the protein.

An advantage of the present method is that it can be used on impure enzymes. The main condition which must be met is that data be obtained under conditions such that the total metal ion concentration be approximately equal to the concentration of free metal ion. This condition can usually be satisfied by the use of a very large excess of the metal ion. If the enzymes are impure this method will give more reliable values of K as the complexes become weaker.

The method presented here can be applied equally well to the inactive complexes formed when a metal inactivates an enzyme. In this case the same kind of data are used. The chief restriction on the use of the method arises from the stability range which can be examined by experiments of this kind. Loose complexes or complexes of moderate stability can be studied very conveniently. Extremely stable complexes would probably not allow reliable stability constants to be obtained. With very stable complexes, the swamping conditions necessary to provide an accurate knowledge of the metal ion concentration would change the enzymic activity to a value so close to its limiting value that the difference could not be measured with any degree of precision. Low concentrations of metal ion (less than enzyme-saturating amounts) would, on the other hand, allow the enzymic activity to fall in a convenient range for study, but the concentration of free metal ion would only be a small fraction of the total metal ion put into the system. In such a case an independent experimental determination of the actual conentration of metal ion in solution would be absolutely necessary. Thus, this method is a convenient one for a large range of stability constants, but is not a completely general method. It is perhaps not too rash to say that none of the methods available for the study of complexes in solution are completely general ones. For very stable complexes few methods are available. It is especially in the examination of these stable complexes, such as $Fe(CN)_6^{3-}$, that precise data have yet to be obtained by any direct method.

Finally, it should be observed that the assumption of the independence of coordination sites is implicit in the commonly used methods for determining enzyme-inhibitor equilibrium constants[7], and, like these, the present method gives information on the interaction of enzymically-active sites and the metal ion. These, of course, may represent only a portion of the total coordinating sites present in the enzyme.

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EAU NON-SOLVANTE ET EAU DE SORPTION DE LA CELLULE DE LEVURE

A. GUILBOT ET A. B. LINDENBERG

Laboratoire de Biochimie Physique et Laboratoire de Biochimie et Physico-chimie des Céréales, Paris (France)

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SUMMARY

Non-solving and adsorbed water in yeast cells

At diffusion equilibrium, the distribution coefficient R of different alcohols and aliphatic amides in the intracellular and extracellular water of baker's yeast is a function of δ, the molar decrement of the dielectric constant, which is defined by these non-electrolytes in water solution: $\ln R = 0.023 \cdot \delta - 0.046$. In this formula, the variable ($0.023\ \delta$) represents the salting-out effect of the salinity in the aqueous cellular medium on the non-electrolytic solutes, while the constant term (-0.046) is a measure of the solving water space available for free diffusion, in the absence of any salting-out effect. Referred to the dry cell substance, this constant indicates the existence of 0.2 g of non-solving water per g of dry yeast.

On the other hand, the adsorption–desorption curves of yeast plotted against the relative humidity of the medium permit the extrapolation of a value of 0.196 g for the quantity of water bound per 1 g of dry cells.

The agreement of results obtained with these two methods of measurement leads to the conclusion that the non-solving water is made up of the total deriving from primary adsorption + absorption in the form ot solid solution in the macromolecules of the yeast cell.

INTRODUCTION

L'un de nous a montré antérieurement que l'on peut évaluer la proportion d'eau non-solvante dans la cellule de levure[1] ou dans le muscle de grenouille[2] par l'étude de la répartition à l'équilibre de diffusion, entre l'eau cellulaire ou tissulaire et l'eau du milieu extérieur, de différents alcools hydrophiles (liquides miscibles à l'eau), très peu solubles dans les constituants cellulaires autres que l'eau.

En effet, le coefficient de répartition (R) $\frac{\text{Eau intra-cellulaire}}{\text{Eau extra-cellulaire}}$ s'avère être constant pour un alcool donné, jusqu'à des concentrations sub-cytolytiques. Mais la valeur de ce rapport, toujours inférieure à l'unité, varie cependant d'un alcool à l'autre, suivant la sensibilité au relargage de la molécule alcoolique par les ions et les molécules présents dans le milieu aqueux cellulaire ou tissulaire.

Or, cette diminution de solubilité due au relargage salin, que les solutés alcooliques subissent dans l'eau solvante intra-cellulaire, est fonction de leur polarité diélectrique molaire dans l'eau. De la sorte, il est possible, connaissant cette fonction, de faire la part de l'influence du relargage salin sur la solubilité différentielle des alcools dans l'eau solvante cellulaire, pour mettre en évidence leur non-diffusibilité dans une fraction déterminée de l'eau totale de la cellule ou du tissu considéré. La quantité d'eau non-solvante que l'on mesure ainsi s'avère être une constante cellulaire, indépendante de la nature de l'alcool diffusant.

Nous avons alors généralisé le résultat obtenu avec les perméants alcooliques, pour admettre l'impénétrabilité absolue d'une certaine proportion de l'eau cellulaire à tout soluté, ionique ou moléculaire, comme à l'eau elle-même.

Cette notion d'eau liée non-solvante, on sait aujourd'hui que'elle est valable même lorsque le substrat avec son eau liée se trouve être dissout dans l'eau. Ainsi, nombre de protéines globulaires (myoglobine, β-lactoglobuline, hémoglobine, sérumalbumine, ovalbumine, conalbumine, ribonucléase, pepsine) présentent en solution aqueuse, au voisinage de leur point isoélectrique, une configuration proche d'une sphère compacte, rigide et impénétrable. Leur viscosité intrinsèque s'écarte cependant d'une manière significative de la viscosité théorique des sphères parfaitement rigides par suite de l'incorporation d'une certaine proportion du solvant au sein des particules protéiques compactes, dissoutes dans l'eau. L'effet de la force ionique sur les courbes de titration de ces solutions étant trop faible pour que l'on puisse admettre la pénétration des ions salins dans l'espace situé entre les charges titrables[3], on est amené à conclure que l'eau incorporée aux particules protéiques dissoutes demeure rigoureusement impénétrable aux ions environnants.

L'étude de ces courbes de titration à différents pH a en outre permis de préciser que les groupements hydrophiles, porteurs de charges titrables, se trouvent à la surface des particules protéiques dissoutes, en contact direct avec le solvant aqueux.

Objet du travail

On sait que, dans un système biologique, c'est la valeur de l'activité thermodynamique, ou de la tension relative de vapeur d'eau, qui permet de définir le plus commodément si l'eau a atteint un degré de liberté suffisamment élevé pour pouvoir intervenir dans une réaction biochimique ou dans une fonction physiologique déterminée.

Or, si la méthode de mesure de répartition à l'équilibre de diffusion permet d'évaluer avec une précision satisfaisante la quantité d'eau non-solvante présente dans un système, elle ne fournit par contre aucune indication quant à l'activité thermodynamique qu'il faut réaliser pour assurer la présence, dans le système considéré, d'une proportion suffisante d'eau solvante pour le déroulement normal de l'activité biologique.

Nous avons ainsi été amenés à procéder à l'étude de la relation entre la pression relative de vapeur d'eau et la teneur en eau de la levure (diagramme de sorption d'eau), pour avoir une idée du mode de fixation de l'eau dans la cellule de levure aux différentes activités thermodynamiques, et pour essayer aussi de préciser le degré d'humidité relative pour lequel l'eau "liée" est encore quasiment seule dans la levure.

MATÉRIAUX ET MÉTHODES UTILISÉS

La levure employée provient de la partie centrale de pains de levure industrielle (Springer), âgés de moins de 12 h. Elle est soigneusement lavée à l'eau distillée et utilisée aussitôt. Pour certains essais, la levure lavée a été ensuite cryodesséchée à une température inférieure à —20°.

Détermination de la teneur en eau: Elle a été effectuée soit par dessiccation jusqu'à poids constant, à 50° sous une pression inférieure à 20 mm de mercure, en présence d'anhydride phosphorique[4], soit par étuvage à 105° à pression normale pendant 20 h. Les deux méthodes fournissent des résultats sensiblements concordants.

Détermination du coefficient de répartition: La méthode utilisée a été décrite[1].

Détermination des isothermes de désorption et de sorption: Pour la désorption, des parties aliquotes de levure lavée et centrifugée (teneur en eau: 70–75 %) sont placées dans des enceintes dont la tension de vapeur d'eau est fixée par des solutions d'acide sulfurique de concentrations différentes, à température constante (22° ± 1°). A l'équilibre, vérifié par la constance de poids, on mesure d'une part la teneur en eau de l'échantillon et d'autre part, par densimétrie, le titre des solutions d'acide sulfurique. Les activités thermodynamiques sont calculées d'après[5].

Pour la sorption, on opère de façon identique, sur des prises d'essai de levure cryodesséchée, dont la teneur en eau est de l'ordre de 3 p. 100 ou a été ramenée à moins de 0,5 p. 100 par séchage ultérieur sous vide à température ordinaire.

RÉSULTATS EXPÉRIMENTAUX

Diminution de solubilité (relargage) des alcools aliphatiques dans l'eau salée, en fonction de leur incrément diélectrique molaire négatif dans l'eau

En solution diluée, la constante diélectrique D est fonction de la concentration C du non-électrolyte, en mol. g/l: $D = Do + \delta C$, Do étant la constante diélectrique de l'eau pure et δ étant l'incrément diélectrique molaire égal à dD/dC. Cet incrément diélectrique est négatif avec tous les alcools aliphatiques même les plus hydrophiles comme le glycérol.

Conformément à la théorie électrostatique du relargage des non-électrolytes par les ions, la solubilité des alcools aliphatiques diminue dans l'eau chargée de sels neutres, d'autant plus fortement que leur incrément diélectrique molaire est plus négatif dans l'eau. Nous avons vérifié que dans une solution molale de chlorure de sodium, par exemple, les coefficients de solubilité (S) des alcools primaires, secondaires et tertiaires se trouvent être abaissés par rapport à leurs coefficients de solubilité dans l'eau pure (So), en suivant la relation: $\log S/So = \log K = 0.035\ \delta$, (20°), la validité de cette relation ayant été vérifiée depuis le méthanol ($\delta = -1.4$) jusqu'au butanol tertiaire ($\delta = -6.3$).

Variation de la constante de répartition $(\mathrm{R}) = \frac{\textit{Eau cellulaire}}{\textit{Eau exterieure}}$ *des alcools en fonction de* δ

Si maintenant, au lieu du milieu aqueux purement salin étudié *in vitro*, on s'adresse au milieu aqueux cellulaire plus complexe de la levure *in vivo*, l'on constate que le logarithme de la constante d'équilibre de répartition R des alcools diffusibles mono-, di- et tri-hydroxylés, entre l'eau totale de la cellule et l'eau pure du milieu extérieur est, ici encore, fonction linéaire du décrément diélectrique molaire (δ) que déterminent ces alcools par leur dissolution dans l'eau. Mais, fait capital, la relation suivie *in vivo* comporte en outre un terme constant indépendant de la nature du soluté diffusant, qui n'apparaît pas dans les systèmes de relargage ternaires eau–ions–nonélectrolyte étudiés *in vitro*.

$$\log R = 0.023 \cdot \delta - 0.046 \quad (1) \qquad \log R/0.9 = 0.023 \cdot \delta \; (20°) \quad (2)$$

TABLEAU I

VARIATION DE LA CONSTANTE D'ÉQUILIBRE DE DIFFUSION (R) DES ALCOOLS ET AMIDES ALIPHATIQUES CHEZ LA LEVURE DE BOULANGERIE EN FONCTION DE DE L'INCRÉMENT DIÉLECTRIQUE MOLAIRE (δ) DE CES NON-ÉLECTROLYTES DANS L'EAU

Les valeurs de R indiquées entre parenthèses correspondent aux moyennes expérimentales, légèrement supérieures à la théorie dans le cas des solutés diffusant dans les constituants lipoïdiques cellulaires d'une manière déjà appréciable.

	δ	*Log R*	R	$R/0.9 = K$
Eau	0	—0.046	0.90	1.0
Méthanol	—1.4	—0.078	0.835 (0.84)	0.928
Glycol	—1.8	—0.0874	0.818	0.910
Glycérol	—2.6	—0.106	0.7835	0.870
Ethanol	—2.65	—0.107	0.78 (0.79)	0.866
Isopropanol	—4.3	—0.145	0.715 (0.735)	0.795
Tert-butanol	—6.3	—0.19	0.646 (0.675)	0.718
Acétamide	—0.8	—0.0644	0.862 (0.875)	0.958
Formamide	+0.8	—0.0276	0.94	1.044
Urée	+2.8	+0.018	1.043	1.16

La constante cellulaire dégagée (—0.046) signifie que log R demeure encore négatif, ($R = 0.9$), même dans le cas d'un soluté hydroxylé non-hydrocarboné, qui ne diminue ni n'augmente la constante diélectrique de l'eau ($\delta = 0$). Autrement dit, l'espace de diffusion libre offert à l'eau elle-même, considérée comme soluté idéal, serait encore limité à une fraction pondérale déterminée, égale aux 0.9ème de l'hydratation totale de la cellule de levure expérimentée.

Sous la forme (2), la relation précédente fait ressortir que la constante de répartition R, qu'on établit en rapportant à l'eau totale cellulaire les quantités d'alcool trouvées à l'équilibre de diffusion à l'intérieur des cellules, n'a pas la signification physicochimique d'un coefficient de partage. Pour ne tenir compte dans le calcul de la concentration alcoolique interne que de la fraction de l'eau cellulaire jouissant des propriétés solvantes, il est nécessaire de diviser R par 0.9. La nouvelle valeur obtenue, $R/0.9 = K$, représente le vrai rapport entre le coefficient de solubilité du non-électrolyte dans l'eau solvante cellulaire, et celui dans l'eau pure extérieure; le logarithme de K redevient de ce fait fonction simple de δ, comme dans un milieu salin *in vitro*.

Notons que cette relation s'applique encore, avec les mêmes paramètres, à des non-électrolytes diffusibles à fonction amide, tels que la formamide, l'acétamide et l'urée, pour lesquels les valeurs expérimentales de R correspondent bien à celles prévues par la théorie sur la base de leurs incréments diélectriques molaires, et de la constante cellulaire (—0.046) dégagée pour les perméants à fonction alcool.

Le résultat obtenu avec l'urée semble particulièrement instructif, en ce qu'il démontre la validité de cette relation pour des solutés plus polaires que l'eau, qui, au lieu de diminuer, augmentent au contraire la constante diélectrique de l'eau (δ positif). Et, en fait, l'augmentation du coefficient de solubilité relative K de l'urée dans l'eau solvante cellulaire (phénomène de "salting-in") s'avère être 1.16 fois plus élevé que dans l'eau pure; la fraction non-solvante de l'eau cellulaire conserve cependant la même valeur dans le cas de l'urée que dans celui des solutés à δ négatif.

Il ressort ainsi clairement que, dans le milieu aqueux cellulaire, on doit tenir compte de la présence: (a) D'ions salins (et d'ions dipolaires), responsables des modifications de solubilité que subissent les molécules perméantes au sein de l'eau solvante. (b) De molécules et surtout macromolécules hydrophiles, qui, comme les protéines et les glucides sont capables de lier assez fortement une partie de l'eau cellulaire, pour la rendre totalement impénétrable à l'égard d'ions et de molécules environnantes, quelle que soit leur polarité.

Ainsi, pour 1 g d'eau cellulaire totale de la levure de boulangerie, on aurait 0.90 g d'eau solvante et 0.10 g d'eau non-solvante. Cependant, l'eau non-solvante ne constitue pas une fraction définie et invariable de l'eau cellulaire totale, mais est fonction de la qualité et de la quantité des constituants cellulaires responsables de la fixation d'une partie de l'eau sous cette forme impénétrable.

Pour donner au résultat précédent une signification physico-chimique plus précise, il convient de l'exprimer par rapport à la substance sèche cellulaire, ce qui conduit à une valeur de 0.20 g d'eau non solvante par g de matière sèche, la levure expérimentée contenant en poids deux parties d'eau intracellulaire pour une partie de substance sèche.

Rappelons qu'en utilisant la même méthode de mesure de l'équilibre de diffusion des alcools hydrophiles, LINDENBERG ET GARY-BOBO[2] ont obtenu, pour le muscle gastrocnémien de grenouille, une teneur en eau non-solvante égale à 0.16 g/g de matière sèche musculaire. La concordance entre ces deux valeurs paraît satisfaisante si l'on considère que la teneur de la levure en protéines, moins élevée que celle du muscle, est compensée par une proportion plus importante de polymères glucidiques hydrophiles.

Notons par ailleurs que, lorsqu'on ne tient pas compte de l'effet de relargage subi par le soluté dans le milieu aqueux cellulaire, la valeur calculée pour l'eau non-solvante est très différente de la valeur réelle: dans le cas de la levure, par exemple, la constante de répartition R de l'éthanol, égale à 0.78, fournirait pour l'eau non-solvante une valeur de 0.22 g/g d'eau cellulaire totale, soit 0.44 g/g de levure sèche, valeur 2.2 fois plus élevée que la proportion réelle.

Isothermes de sorption et de désorption de la levure de boulangerie

Dans le Tableau II sont rassemblées les activités thermodynamiques et les teneurs en eau correspondantes, à 22 ± 1°, pour deux échantillons de levures en désorption et un échantillon de levure en sorption*.

En portant en abcisse l'activité de l'eau et en ordonnée les quantités d'eau fixées par gramme de matière sèche, on obtient les courbes de sorption et de désorption de la levure (Figs. 1 et 2).

La concordance entre les résultats de désorption obtenus à partir de deux préparations de levure différentes est remarquable et permet de tracer une courbe unique.

En sorption, les résultats obtenus avec des échantillons ayant respectivement

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* Résultats obtenus avec la collaboration de Mlle MERCIER.

TABLEAU II

HYDRATATION DE LA LEVURE EN FONCTION DE LA PRESSION RELATIVE (ACTIVITÉ THERMODYNAMIQUE) DE VAPEUR D'EAU DANS LE MILIEU ENVIRONNANT

Désorption				*Sorption*	
Préparation 1		*Préparation 2*		*Préparation 1*	
Activité thermodyn.	*H_2O/g de substance sèche*	*Activité thermodyn.*	*H_2O/g de substance sèche*	*Activité thermodyn.*	*H_2O/g de substance sèche*
0.018	0.0251			0.018	0.0202*
0.038	0.0331			0.038	0.0299*
0.052	0.0472	0.066	0.0494	0.052	0.0395
0.154	0.0629	0.154	0.0651	0.162	0.0580
0.280	0.0842	0.274	0.0815	0.282	0.0774
		0.376	0.0998	0.333	0.0843*
0.626	0.1587	0.628	0.1598	0.384	0.0949
0.736	0.2150	0.738	0.2286	0.528	0.1226*
0.862	0.3739	0.872	0.423	0.628	0.1534
0.946	0.8770	0.946	0.845	0.628	0.1582*
				0.728	0.2175
				0.730	0.2164
				0.796	0.2764
				0.852	0.3844
				0.926	0.6802

* Prises d'essai de teneur en eau initiale inférieure à 0.5%.

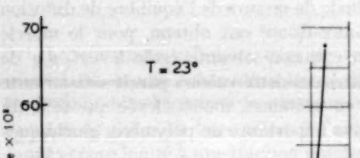

Fig. 1. Sorption de l'eau par la matière sèche de levure en fonction de l'activité de vapeur d'eau dans le milieu environnant.

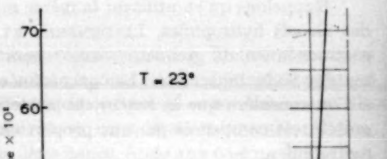

Fig. 2. Désorption de la levure hydratée en fonction de l'activité de vapeur d'eau dans le milieu environnant.

une teneur en eau initiale inférieure à 0.5 % et à 3.5 %, ne présentent pas de différence significative et se placent sur une même courbe.

Du reste, le phénomène d'hystérésis est assez faible, dans la région d'activités située entre 0.05 et 0.60; l'écart observé entre les teneurs en eau de sorption et de désorption pour une même activité est inférieur à 0.01 g, en valeur absolue.

DISCUSSION

Ces courbes présentent la forme classique en *S*, typique de la fixation d'eau par les colloïdes biologiques; elles sont généralement interprétées comme décrivant deux phénomènes qui se succèdent en se superposant plus ou moins:

– dans la région des faibles activités d'eau, une adsorption primaire en couche monomoléculaire, pour laquelle les forces de liaisons entre l'eau et le substrat sont très fortes;

– puis un phénomène de sorption secondaire d'allure accélérée. Ces deux phénomènes peuvent être sommairement représentés par l'équation de BRUNAUER, EMMETT ET TELLER[6] qui implique la possibilité d'une adsorption multimoléculaire et rend compte souvent des observations expérimentales qui dévient grandement des types de courbes donnés par l'équation logarithmique de FREUNDLICH, ou l'équation hyperbolique de LANGMUIR.

Mais, en fait, les segments de courbes dans la région d'activités de 0.06 à 0.40 se présentent comme des portions de droites coupant l'axe des ordonnées en un point équivalent à 0.031 g d'eau/g de levure sèche, dans le cas de la sorption et 0.04 g dans le cas de la désorption.

Ces droites ont respectivement pour équations: (III) $g = 0.165\, a + 0.031$ et (IV) $g = 0.156\, a + 0.04$, g étant le nombre de gramme d'eau fixé par g de matière sèche cellulaire et a l'activité thermodynamique de l'eau en équilibre avec la levure (Tableau III).

Dans notre interprétation, les constantes 0.031 et 0.04 correspondent à l'eau adsorbée, respectivement en cours de sorption et de désorption, à la surface des

TABLEAU III

L'HYDRATION DE LA LEVURE EN TANT QUE FONCTION LINÉAIRE DE L'ACTIVITÉ DU VAPEUR D'EAU, DANS LA RÉGION D'ACTIVITÉS MODÉRÉES

Désorption			*Sorption*		
Activités thermodynamiques = a	*g mesuré*	*g calculé selon IV*	*Activités thermodynamiques = a*	*g mesuré*	*g calculé selon III*
0.052	0.0472	0.0481	0.052	0.0395	0.0396
0.066	0.0494	0.0503	0.162	0.0580	0.0577
0.154	0.0629	0.0640	0.282	0.0774	0.0775
0.154	0.0651	0.0640			
0.274	0.0815	0.0827	0.333	0.0843	0.0859
0.280	0.0842	0.0837	0.384	0.0949	0.0944
0.376	0.0998	0.0986			
			0.528	0.1226	0.1181
0.626	0.1587	0.1376	0.628	0.1534	0.1345
0.628	0.1598	0.1379		0.1582	

constituants macromoléculaires de la levure. L'adsorption de l'eau tend, dans chacun des cas, vers une valeur limite (A_∞) pour une activité encore très faible, de l'ordre de 0.06, en raison de la très grande valeur numérique de la constante a de l'équation hyperbolique de LANGMUIR $g = \frac{A_\infty}{1/a + a} \cdot a$. Autrement dit, par rapport à 0.06, $1/a$ peut être considéré comme négligeable et la relation peut s'écrire: $g \simeq A_\infty$.

Cependant, l'adsorbant cellulaire "dissout" de l'eau, à la faveur d'une activité thermodynamique croissante, comme il le fait d'ailleurs depuis le début de la sorption, en obéissant à la loi de proportionalité de HENRY. Cette eau forme une solution solide dans le gel macromoléculaire de la levure; elle devient impénétrable aux solutés environnants, les forces de coordination, de contrainte, et de tassement existant dans cette solution solide étant supérieures à celles intervenant dans l'eau à l'état liquide. Il est permis de penser que ce processus continue jusqu'à des activités voisines de l'unité.

Des interprétations quelque peu analogues ont déjà été proposées, par PIERCE[7] pour la sorption de l'eau par le coton et par HAILWOOD ET HORROBIN[8], dont NORTHCOTE[9] a utilisé l'équation pour l'étude des phénomènes observés avec les polyholosides constituant la paroi cellulaire de la levure.

Quoiqu'il en soit, à l'activité unitaire, les deux équations linéaires correspondant respectivement à la sorption et à la désorption de la levure fournissent la même valeur: 0.196 pour la quantité d'eau fixée à 1 g de matière sèche. Ce résultat indique que le retard observé dans la fixation d'eau au cours de la sorption en fonction de l'activité (phénomène d'hystérésis) porte surtout sur la fraction d'eau correspondant à la première couche et est complètement rattrapé à l'activité = 1.

Mais le fait le plus remarquable est la concordance entre la quantité d'eau "fixée" ainsi définie (0.196) et celle de l'eau non-solvante (0.2) déterminée par l'étude de la diffusion d'équilibre des non-électrolytes hydrophiles. Ceci nous autorise à identifier l'eau non-solvante comme étant la somme: eau d'adsorption primaire + eau en état de solution solide dans les macromolécules de la cellule de levure.

Au-delà de l'activité = 0.45, on constate que les courbes de sorption et désorption commencent à s'écarter des droites que l'on vient de définir, et s'incurvent sous l'effet de la solvatation, tendant à la limite vers les lois des solutions diluées. La levure commence ainsi à retenir des quantités de plus en plus considérables d'eau solvante, susceptible d'intervenir dans les réactions biochimiques. Cependant il est vraisemblable que l'activité biologique se développant dans cette région des moyennes tensions relatives de vapeur d'eau soit encore très limitée, en raison de la faible proportion d'eau solvante présente dans la cellule. Et, en fait, d'après BURCIK[10], le minimum d'humidité relative nécessaire à la multiplication de *Saccharomyces Cerevisiae* est de 0.895.

CONCLUSIONS

L'examen des courbes de sorption–désorption de la levure de boulangerie permet de préciser que:

- 0.031 g d'eau/g de matière sèche en sorption et 0.04 g en désorption peuvent être considérés comme répondant à l'équation d'adsorption de LANGMUIR et retenus à la surface des constituants macromoléculaires des cellules par liaison avec des groupements polaires.

– une fraction d'eau (g) formant une solution solide dans le gel macromoléculaire de la levure obéit à la loi de HENRY et peut être représentée en fonction de l'activité thermodynamique (a) par $g = 0.165\ a + 0.031$ et $g = 0.156\ a + 0.04$ respectivement en sorption et désorption.

– à partir d'une activité de 0.45–0.50, les courbes s'écartent des droites ainsi définies et la levure fixe une proportion d'eau de plus en plus importante par solvatation.

La confrontation des résultats obtenus par la méthode de diffusion d'équilibre des non-électrolytes hydrophiles et par la méthode de sorption permet d'identifier l'eau non solvante à la somme "eau d'adsorption primaire + eau absorbée selon la loi de HENRY jusqu'à l'activité = 1" dans les macromolécules de la cellule de levure. Cette somme est identique en sorption et en désorption et correspond à 0.196 g/g de matière sèche.

Ce n'est qu'au delà d'une tension relative de vapeur d'eau de 0.45–0.50 que l'activité biologique de la levure peut commencer à se manifester.

RÉSUMÉ

A l'équilibre de diffusion, le coefficient de répartition R de différents alcools et amides aliphatiques entre l'eau intra-cellulaire et l'eau extra-cellulaire de la levure de boulangerie est fonction du décrément diélectrique molaire δ que déterminent ces nonélectrolytes en solution diluée dans l'eau: $\log R = 0.023\ \delta - 0.046$. Dans cette formule, le terme variable ($0.023\ \delta$) représente l'effet de relargage excercé par la salinité du milieu aqueux cellulaire à l'égard des solutés non-électrolytiques, le terme constant (-0.046) mesurant l'espace aqueux solvant offert à la diffusion libre en l'absence de tout effet de relargage. Par rapport à la substance sèche cellulaire, cette constante indique l'existence de 0.2 g d'eau non-solvante par g de matière sèche levurienne.

D'autre part, les courbes de sorption–désorption de la levure en fonction de l'humidité relative du milieu permettent d'extrapoler une valeur de 0.196 g pour la quantité d'eau fixée à 1 g de matière sèche cellulaire.

La concordance entre ces deux catégories de mesures nous autorise à identifier l'eau non-solvante comme étant constitutée par la somme eau d'adsorption primaire + eau d'absorption en état de solution solide dans les macromolécules de la cellule de levure.

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STUDIES OF THE BACTERIAL CELL WALL

VI. WALL COMPOSITION AND SENSITIVITY TO LYSOZYME

M. R. J. SALTON AND J. G. PAVLIK
Department of Bacteriology, University of Manchester (Great Britain)

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SUMMARY

All of the isolated walls of the Gram-positive bacteria investigated showed some degree of susceptibility to digestion with egg-white lysozyme; the % Turbidity reduction varied from 9 % for *Staphylococcus aureus* walls to 98 % for the walls of *Micrococcus lysodeikticus*. Although all of the walls contained muramic acid and glucosamine, the sensitivity to lysozyme was not correlated with the total amino sugar contents. The nature of the monosaccharide constituents and the relative proportions of amino acids have been determined. Walls of a number of organisms contain peptides of fairly similar composition. Several unidentified compounds have been detected in walls of various species.

Only *Lactobacillus arabinosus* walls showed an increased sensitivity to lysozyme after removal of O-ester groups or the teichoic acid. Apart from substituent groups, one likely explanation of differences in sensitivity to lysozyme seems to be the proportion of N-acetylamino sugars linked in a specific manner.

INTRODUCTION

The substrate of egg-white lysozyme is known to be distributed throughout both the Gram-positive and Gram-negative groups of bacteria[1-5]. Within the Gram-positive group, organisms vary in their susceptibility to lysis by lysozyme, from the most sensitive species *Micrococcus lysodeikticus*, to the more resistant staphylococci, streptococci and other genera. As the "substrate" constitutes part of the peptide-amino sugar complex of the bacterial cell wall[6] an investigation of the factors governing the lysozyme sensitivity of isolated walls of a number of Gram-positive organisms was undertaken. The chemical constitution of the walls showing different susceptibilities to digestion with lysozyme was studied to determine whether any features of composition could be correlated with response to treatment with lysozyme. Since the commencement of the investigations reported in this paper, BRUMFITT, WARDLAW AND PARK[7] have shown that the lysozyme resistance of a mutant strain of *M. lysodeikticus* was due to the presence of O-acetyl groups. The relevance of this finding to the general problem of sensitivity to lysozyme will be discussed.

Abbreviations: DAP, α,ε-diaminopimelic acid; TCA, trichloroacetic acid.

MATERIALS AND METHODS

Organisms and preparation of cell walls

Cell walls were prepared from the following organisms, which unless specified were either laboratory strains or strains used in previous studies: *Bacillus cereus* (P2); *Bacillus megaterium* (KM); *Bacillus pumilis* (NCTC 6353); *Bacillus stearothermophilus* (2184), kindly provided by Dr. C. Georgi; *Bacillus subtilis*; *Bacillus thuringiensis*, kindly provided by Dr. R. Munro; *Bacillus* species (probably a variant of *Bacillus sphaericus*, strain isolated from soil by D. Wiseman); *Corynebacterium* species, kindly provided by Dr. H. L. Jensen; *Lactobacillus arabinosus* (17/5); *Micrococcus citreus* (NCTC 7731); *Micrococcus lysodeikticus* (NCTC 2665); *Micrococcus roseus*; *Micrococcus tetragenus*, kindly provided by Dr. A. Linton; *Micrococcus urea*; *Micrococcus varians* (NCTC 7281); *Sarcina lutea*; *Sporosarcina urea*; *Staphylococcus albus* (NCTC 7944); *Staphylococcus aureus* (Duncan); *Staphylococcus citreus*; *Staphylococcus saprophyticus*; *Streptococcus faecalis* (NCTC 6782). All organisms were grown on nutrient agar or nutrient broth, with the exception of *L. arabinosus* and *Corynebacterium* sp. (both grown on a 2 % Difco Casitone medium, supplemented with 0.1 % Marmite) and *Strep. faecalis* (grown in nutrient both with 1 % glucose and 0.1 % Marmite). Organisms were grown at 30°, except for *B. stearothermophilus* (55°); *M. citreus*, *M. varians* and *Sp. urea* (25–28°); *Bacillus* sp. (20°).

The cells were generally harvested in the stationary phase of growth, washed with distilled water and disintegrated in the MICKLE apparatus and the walls isolated as previously described[8]. The wall preparations were finally digested with trypsin and washed free of salts prior to freeze-drying or storage as concentrated suspensions.

Lysozyme sensitivity tests

Walls were digested with crystalline, egg-white lysozyme (Armour Laboratory) in phosphate buffer of ionic strength (μ) = 0.1, pH 7.0, at 37°, under the conditions used previously[6]. Lysozyme was used at a final concentration of 50 μg/ml. The dissolution of walls was followed in a Unicam spectrophotometer (Model SP 600) at a wavelength of 700 mμ. The % turbidity reduction was obtained from: [(initial O.D. — final O.D.)/initial O.D.] × 100. The initial turbidities of the wall suspensions were adjusted so that an $E_{1\,\mathrm{cm}}$ of 0.7 at 700 mμ corresponded to a suspension containing approximately 2 mg dry wt. cell walls/ml.

Chemical analyses and paper chromatographic techniques

Estimation and identification of monosaccharide constituents: Reducing substances and polysaccharide contents were estimated by the method of Hagedorn and Jensen (modified by Hanes[9]), and the anthrone method described by Seifter, *et al.*[10]. Glucose was determined specifically with glucose oxidase by the procedure of Keilin and Hartree[11].

The sugars were identified by hydrolyzing wall preparations for 12–16 h at 100° in 0.5 *N* H_2SO_4, neutralizing with $Ba(OH)_2$ to pH 4.5, de-ionizing and examining the hydrolysates on Whatman paper No. 1, after descending chromatography with solvent of *n*-butanol–pyridine–water/(6:4:3, v/v). The papers were sprayed with aniline phthalate.

Amino sugars

Wall preparations were hydrolyzed in sealed ampoules and the amino sugar contents determined by the method of RONDLE AND MORGAN[12]. The detection of amino sugars on paper chromatograms was achieved by two-dimensional separation of hydrolysis products on Whatman paper No. 1 with pyridine–water (4:1, v/v) in one direction and *n*-butanol–acetic acid–water (6:1:2, v/v) as the second solvent. Under these conditions both glucosamine and galactosamine were separated from muramic acid and all three amino sugars showed a good separation from the amino acids. The identity of muramic acid in the cell walls was checked in several cases against a synthetic sample of muramic acid kindly provided by Dr. O. TOUSTER; this was performed by electrophoresis on Whatman paper 3MM, 0.05 M $Na_2B_4O_7$ buffer, pH 9.2, for 2 h at 10 V/cm.

Amino acids

For the identification of amino acids, cell walls were hydrolyzed with 6 N HCl, 16 h, 105° and the products examined by two-dimensional paper chromatography using the solvent systems used for the separation of amino sugars (see above). Quantitative estimations of amino acids were also performed after separation on Whatman paper No. 1, with the pyridine–water, butanol–acetic acid–water solvents. After steaming, the papers were dipped in a 5 % w/v solution of ninhydrin in acetone containing 5 % by volume of 0.05 M phosphate buffer (pH 7), developed and extracted as previously described[13]. The ninhydrin colours were estimated at 570 mμ. Control samples of amino acids were similarly treated to determine differences in colour yields. Only glycine gave half the colour yield obtained with the other amino acids and a correction factor has been applied. Triplicate chromatograms were treated in this way for each cell wall preparation.

Quantitative estimations of lysine were performed by the colorimetric method of CHINARD[14] and the estimation of DAP was carried out as described by WORK[15].

The isomers of DAP were identified after paper chromatographic separation with the solvent system of RHULAND, *et al.*[16].

Ribitol phosphate compounds

Walls were hydrolyzed under the conditions suggested by BADDILEY, *et al.*[17] (2 N HCl, 3 h at 100°). Hydrolysates prepared for amino acid identification (6 N HCl, 16 h, 105°) were also examined. Descending chromatograms on Whatman paper No. 1, using *n*-butanol–acetic acid–water (3:1:1, v/v), and ascending chromatograms with *n*-propanol–ammonia (d. 0.88)–water (6:3:1, v/v) were prepared and sprayed according to the method of BUCHANAN, DEKKER AND LONG[18].

O-ester groups

The method of HESTRIN[19] was used for the estimation of O-ester groups. After reaction of the walls with the hydroxylamine reagent[19], the preparations were acidified and the walls removed by centrifugation to give clear supernatant fluids for the estimation of the hydroxamates. Acetylcholine was used as a standard and results have been expressed in terms of O-acetyl groups although we have not specifically confirmed that the O-ester contents are due solely to O-acetyl groups.

EXPERIMENTAL

Sensitivity of cell walls to dissolution with lysozyme

Fig. 1 shows that the isolated walls of various bacteria differ widely in their sensitivity to lysozyme, as indicated by both the initial speed of reduction of turbidity and the final level of turbidity reduction. Measurements of the turbidity reduction after 24 h and 48 h showed that little change occurred during the 24–48 h period of incubation with the enzyme. All subsequent determinations of "sensitivity" have therefore been performed by measuring the extent of digestion (expressed as % turbidity reduction) after 24 h incubation with lysozyme.

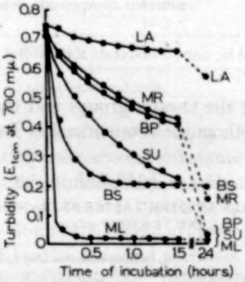

Fig. 1. The action of lysozyme on cell walls of *LA* (*Lactobacillus arabinosus*); *MR* (*Micrococcus roseus*); *BP* (*Bacillus pumilis*); *BS* (*Bacillus subtilis*); *SU* (*Sporosarcina ureae*); *ML* (*Micrococcus lysodeikticus*). Digestion of walls with 50 μg lysozyme/ml, pH 7 at 37° followed by measuring turbidity changes at 700 mμ.

Effect of extraction of O-ester groups and the teichoic acids on sensitivity to lysozyme

To determine what influence the removal of O-ester groups and extraction of the ribitol phosphate polymer (the teichoic acids of bacterial walls, ARMSTRONG *et al.*[20]), had on the sensitive of the walls to lysozyme, cell-wall suspensions from several bacteria were treated as follows: (a) control, unextracted preparations; (b) extracted with 0.01 *N* NaOH for 1 h at 37°; (c) extracted with 10 % w/v TCA for 24 h at 4°; (d) extracted with 10 % w/v TCA for 5 min at 100°. After extraction the walls were deposited by centrifugation and washed 4 times with distilled water to remove the extracting reagents and the extractable products. The cell-wall residues were suspended in buffer and the extent of turbidity reduction determined.

The cell walls extracted in the above manner were examined for their O-ester contents and the products of hydrolysis were separated for the detection of ribitol compounds. The results are presented in Table I. The presence of teichoic acid was indicated by the detection of ribitol and anhydroribitol in the 2 *N* HCl hydrolysates. Hydrolysis of walls with 6 *N* HCl gave a new compound (R_F anhydroribitol of 1.5 in *n*-butanol–acetic acid–water) not previously reported by ARMSTRONG *et al.*[20]. It is likely that this compound is a dianhydroribitol since samples of ribitol and anhydroribitol heated in sealed ampoules in 6 *N* HCl for 16 h at 105° also gave rise to a compound with the same chromatographic behaviour on paper. Unknown compounds giving a purple-blue colour were observed near the origin on chromatograms of some hydrolysates.

TABLE I

O-ESTER CONTENTS AND RIBITOL COMPOUNDS DETECTED IN CELL WALLS

Walls from	*O-ester contents % dry wt. walls (expressed as O-acetyl)*	*Detection of ribitol compounds with Schiff's spray reagent*
M. lysodeikticus	0.008	None detectable in 30 mg wall
B. cereus	0.02	R*, AR* trace amounts; unknown compounds detected
B. subtilis	0.25	R, AR, RP; dianhydroribitol (?) in 6 *N* HCl hydrolysates
L. arabinosus	0.84	R, AR, RP; dianhydroribitol (?) in 6 *N* HCl hydrolysates
M. roseus	0.43	None detectable in 30 mg wall; unknown compounds detected
Strep. faecalis	0.78	R, AR, RP; dianhydroribitol (?) in 6 *N* HCl hydrolysates; unknown compounds detected

* Abbreviations: R, ribitol; AR, anhydroribitol; RP, ribitol phosphate.

The effects of removing the O-ester groups and the ribitol phosphate polymers from the walls that contain them are summarized in Table II.

TABLE II

DISSOLUTION OF WALLS BY LYSOZYME AFTER EXTRACTION OF O-ESTER GROUPS* AND TEICHOIC ACIDS**

Walls from	*% turbidity reduction after 24 h digestion at 37°*			
	Control	*0.01 N NaOH 1 h, 37°*	*10% w/v TCA 16 h, 4°*	*10% w/v TCA 5 min, 100°*
M. lysodeikticus	96	95	93	—***
B. cereus	23.5	7.3	5.6	1.9
B. subtilis	67	74	56	—***
L. arabinosus	30	97	70	85
M. roseus	65	65	39	45
Strep. faecalis	73	76	65	56

* With NaOH.

** With TCA (trichloroacetic acid).

*** Walls almost completely solubilized under these conditions; insufficient hot TCA-insoluble residues left for testing.

Estimation of the polysaccharide, amino sugar and amino acid constituents of the walls

Polysaccharide: In previous studies the "polysaccharide" contents of the walls were determined by measuring the reducing values of hydrolyzed preparations[21]. These investigations[21] indicated that maximum reducing values were obtained on hydrolysis for 2 h with 2 *N* HCl at 100°. To ascertain whether the HAGEDORN AND JENSEN method gives a valid estimate of the amounts of reducing substances in the walls, determinations were also made by the anthrone procedure and glucose contents measured by the glucose oxidase method. For this purpose, walls of *M. lysodeikticus*, *S. lutea* and *B. megaterium* were selected as glucose was the only reducing substance detected, other than the amino sugars[6]. The results for individual wall preparations are presented in Table III.

The HAGEDORN AND JENSEN method gave values considerably higher than those expected from the summation of the glucose and amino sugar contents; the anthrone

TABLE III

A COMPARISON OF CELL-WALL "POLYSACCHARIDE" CONTENTS DETERMINED BY THREE DIFFERENT METHODS

Walls from	"*Polysaccharide*" *contents as % dry wt. cell walls*			
	Batch No.	*Reducing value** *(Hagedorn & Jensen)*	*Anthrone**	*Glucose oxidase*
B. megaterium	1	45.6	19.4	13.7
	2	40.4	12.1	9.4
M. lysodeikticus	1	42.6	7.5	3.5
	2	43.1	7.5	4.3
	3	44.7	—	5.8
S. lutea	1	42.5	17.1	9.5
	2	42.2	15.0	8.5

* Results expressed in terms of glucose standards.

method underestimated the total quantity of reducing substances. When the SOMOGYI[22] method was used for estimating the reducing substances in the walls of *S. lutea* very little difference from the values obtained by the HAGEDORN AND JENSEN method was apparent (compare values in Table III with 35–42 % for SOMOGYI method). It is probable that interference due to the amino acids[23] liberated on hydrolysis, results in the high values obtained by the reducing methods. In view of this evidence no further attempt was made to determine the "polysaccharide" contents of walls used in this investigation.

Amino sugars

The hydrolysis conditions necessary for the maximum liberation of amino sugars in the cell walls of *M. lysodeikticus* were investigated by hydrolyzing with 1 *N*, 2 *N*, 4 *N* and 6 *N* HCl for various periods of time at 100°. The HCl was removed *in vacuo* and the amino sugar contents determined after neutralizing the hydrolysates. The results given in Table IV indicate a maximum liberation with 6 *N* HCl in 2 h at 100° with a subsequent destruction of 25 % of the amino sugar content on further hydrolysis for a total of 20 h. Although 6 *N* HCl gave the maximum values for *M. lysodeikticus*, the removal of HCl *in vacuo* from a large number of samples presented some difficulty. For the comparative studies presented here it was felt that hydrolysis with 2 *N* HCl for 2 h at 100° would provide an adequate measure of the relative contents of amino sugars; all subsequent determinations were performed under these conditions.

TABLE IV

EFFECT OF HYDROLYSIS CONDITIONS ON THE AMINO SUGAR CONTENT OF THE WALL OF *M. lysodeikticus*

Normality of hydrochloric acid	*Amino sugar content as % dry wt. walls*				
	Hours of hydrolysis at 100°				
	1	2	5.25	12	20
1 *N*	17.5	18.8	19.0	17.0	16.4
2 *N*	19.2	19.0	19.0	17.8	18.0
4 *N*	19.8	20.8	—	17.8	17.5
6 *N*	22.0	24.0	22.2	19.0	17.7

Amino acids

To determine suitable hydrolysis conditions for the release of amino acids, cell walls were hydrolyzed in sealed ampoules with 4 *N* HCl and 6 *N* HCl at 105°. The HCl was removed *in vacuo* and the release of lysine from the walls of *S. lutea* and DAP from *M. varians* walls was determined colorimetrically. The results are presented in Fig. 2. For all subsequent determinations of the proportions of amino acids in the walls, hydrolysis has been performed for 16 h with 6 *N* HCl at 105°.

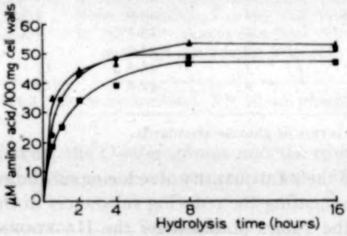

Fig. 2. Liberation of amino acid from cell walls on hydrolysis with HCl at 105°. Release of lysine from walls of *S. lutea* with 4 *N* HCl (■) and 6 *N* HCl (▲); release of DAP from walls of *M. varians* with 6 *N* HCl (●).

RESULTS

The results of an examination of the sensitivity of the walls to lysozyme, the determination of amino sugar contents and the monosaccharide constituents identified by paper chromatography are presented in Table V. In addition to the monosaccharide constituents listed in Table V, the amino sugars separated on paper chromatograms indicated the presence of glucosamine and muramic acid in all of the walls studied. Only a faint trace of muramic acid was detectable in the wall of the *Corynebacterium* sp. Although the identity of the glucosamine "spots" and the muramic acid "spots" on the paper chromatograms was checked by examination of the sugars formed by degradation with ninhydrin, the method does not permit the differentiation of glucosamine from mannosamine nor detection of similar structural differences, if they exist, for muramic acid. However, electrophoresis of hydrolysates of *M. lysodeikticus*, *B. megaterium* and *B. cereus* walls on paper with borate buffer showed compounds corresponding to glucosamine and to one of the two compounds present in the synthetic sample of muramic acid.

The principal amino acid constituents of the bacterial walls have been identified and quantitative estimations have been performed. The relative molecular proportions of the amino acids in the various walls are summarized in Tables VI and VII. It should be emphasized that the amino acids recorded in Tables VI and VII represent the main amino acid compounds obtained on acid hydrolysis. In many instances very little other than the principal amino acids were present but in other walls smaller amounts of amino acids such as aspartic acid, threonine, leucine/isoleucine, and valine have been detected. An unidentified ninhydrin-reacting substance has been found in the wall of *B. pumilis*.

TABLE V

LYSOZYME SENSITIVITY, AMINO SUGAR CONTENTS AND MONOSACCHARIDE CONSTITUENTS OF CELL WALLS

Abbreviations: Gl., glucose; Gal., galactose; Man., mannose; Rh., rhamnose; "X", unknown compounds; (tr.), trace amounts.

Walls from	*Lysozyme sensitivity % turbidity reduction (24 h, 37°)*	*% amino sugar (2 N HCl, 2 h at 100°)*	*Monosaccharide constituents detected*
B. cereus	52 (24)*	31	Gl.
B. cereus var. *mycoides*	51	32	Gl.
B. megaterium	95	18	Gl.
B. pumilis	95	9.2	Gl. (tr.)
Bacillus sp.	84	6.7	Gl.
B. stearothermophilus	80	12.7	Gl., Gal.
B. *subtilis*	69	6.8	Gl.
B. thuringiensis	40 (15)*	9.8	Gl., Man. (tr.)
Corynebacterium sp.	92	14.3	Gal., Rh.
L. arabinosus	22	11.5	Gl., Gal., Rh.
M. citreus	72	21.7	Gl., "X"
M. lysodeikticus	98	16–22	Gl.
M. roseus	75	16	Gal.
M. tetragenus	85	13	Gl., Gal. (tr.)
M. urea	96	19	Gl.
M. varians	93	10	Gl., Gal., "X"
S. lutea	98	12–16	Gl.
Sporosarcina ureae	98	14	None
Staph. albus	13	16.8	Gl.
Staph. aureus	9	17	Gl., Gal. (tr.)
Staph. citreus	32	10	Gl.
Staph. saprophyticus	91	14	Gl., Gal., (tr.)
Strep. faecalis	73	22	Gl., Gal., Rh.

* Batches of walls isolated from the same organism showing different lysozyme sensitivities.

TABLE VI

RELATIVE MOLECULAR PROPORTIONS OF THE PRINCIPAL AMINO ACIDS IN CELL WALLS

Walls from	*Lysine*	*Glutamic acid*	*Glycine*	*Serine*	*Alanine*
Bacillus sp.*	1	1.7	0.5	0.3	2.3
Corynebacterium sp.	1	1	0**	0.7	3.9
M. citreus	1	3	0.8	0	2.1
M. lysodeikticus	1	1	1	0	2.6
M. roseus	1	1.1	0	0	5.1
M. tetragenus	1	1.2	1.2	0	2.3
M. urea	1	1.3	1	0	2.3
S. flava	1	1.4	1	0	2.2
S. lutea	1	1.6	1	0	2.0
Sp. ureae	1	1.7	1	0	2.0
Staph. albus	1	1.1	4.8	0.8	2.9
Staph. aureus	1	1.1	4.8	0.9	3.0
Staph. citreus	1	1	4	1	3.1
Staph. saprophyticus	1	1	4.6	1.2	3.3
Strep. faecalis	1	0.9	0	0	4

* A high proportion of threonine (0.7) was also present.

** "O" used to designate absence or only faint traces of amino acids.

TABLE VII

RELATIVE MOLECULAR PROPORTIONS OF THE PRINCIPAL AMINO ACIDS IN CELL WALLS

Walls from	*DAP**	*Glutamic acid*	*Glycine*	*Alanine*
B. cereus	1	1.3	0	2.6
B. megaterium	1	1.8	0	2.8
B. pumilis	1	1.6	0	4.6
B. stearothermophilus	1	2.0	0	3.8
B. subtilis	1	2.4	0	4.3
B. thuringiensis	1	1.4	0	2.8
M. varians	1	4.3	1.8	2.6
L. arabinosus	1	1.1	0	2.9

* α,ε-diaminopimelic acid.

The results of cell-wall composition of certain of the organisms studied in this investigation are in general agreement with some of the details reported by other investigators[25–28].

DISCUSSION

All of the isolated walls of the Gram-positive bacteria used in this investigation have shown some degree of sensitivity to lysozyme. Some variations in sensitivity of walls to lysozyme has been experienced for different batches of walls of two organisms, *B. cereus* and *B. thuringiensis*. In addition *Strep. faecalis* reported as lysozyme resistant several years ago (SALTON[29]) has under the present conditions of cultivation proved to be sensitive (73 % turbidity reduction). LITWACK[30] has reported that growth conditions alter the sensitivity of intact cells of *M. lysodeikticus* to lysozyme. The mechanisms involved in these changes in sensitivity to lysozyme are not understood. Although the major cell-wall constituents do not appear, at least qualitatively, to be influenced by the growth medium, it is possible that growth conditions may have a profounder effect on the detailed structure of the cell-wall polymer.

Although lysozyme liberates acetylamino sugar substances from the bacterial wall[6], sensitivity to the enzyme is not correlated with the total amino sugar content (see Table V). However, it is evident that the ratios of glucosamine to muramic acid differ for various walls (SALTON, unpublished observations), so the total amino sugar content gives no indication of the possible number of sensitive bonds.

The recent observations of BRUMFITT, *et al.*[7] have established that O-acetyl substituents in the wall can govern the sensitivity of *M. lysodeikticus* to lysozyme. Of the organisms extracted for removal of O-ester groups in this investigation, only *L. arabinosus* walls showed an increased sensitivity to digestion with lysozyme. Procedures extracting the teichoic acids from the walls of this organism also resulted in an increased susceptibility but it is not known whether this effect is due solely to the removal of the teichoic acids or whether O-ester groups are simultaneously removed from the lysozyme "substrate".

While the presence of O-ester groups (presumably attached to the N-acetylamino sugars) is now known to be responsible for the resistance of the walls of two organisms, our results make it evident that this factor cannot account for the differences in the sensitivity of the walls of many organisms used in this investigation. The presence of substituent groups other than O-acetyl or O-ester groups may also account for some of the differences in lysozyme sensitivity. The recent report of a "protein bound' 6-phosphoryl muramic acid by Agren and De Verdier[31] has raised the possibility of the existence of this compound as a wall constituent. If such a compound was present it may render the wall less sensitive to lysozyme due to the presence of the phosphoryl group. A preliminary search for such a compound in the wall of *B. cereus* has so far been unsuccessful.

The results of the present studies could be explained by the existence of differences in the mode of linkage of the acetylamino sugars to one another, thereby determining the number of specific bonds sensitive to lysozyme. If it is assumed that lysozyme is a 1,4-β-N-acetylhexosaminidase and that the walls may have 1,4, 1,6 and/or 1,3-β-glycosidic bonds between the N-acetylamino sugars, then the presence of a high proportion of 1,4 bonds between N-acetylmuramic acid and N-acetylglucosamine would make the wall more sensitive to lysozyme than one containing a high proportion of 1,6 or 1,3 linkages. The possibility that lysozyme sensitivity may be governed by the relative proportions of N-acetylamino sugars linked in the several different possible ways is under investigation.

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ISOLATION OF 6-HYDROXYOESTRADIOL-17β AND OESTRIOL AFTER INCUBATION OF OESTRADIOL-17β WITH RAT-LIVER SLICES

H. BREUER AND R. KNUPPEN

Chemische Abteilung, Chirurgische Universitätsklinik, Bonn-Venusberg (Germany)

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SUMMARY

Following the incubation of oestradiol-17β with liver slices of male rats two metabolites more "polar" than oestradiol-17β were isolated and conclusively identified as 6-hydroxyo estradiol-17β and oestriol.

INTRODUCTION

In 1949, PEARLMAN AND DEMEIO[1] incubated oestradiol-17β hemisuccinate with rat-liver slices and obtained a comparatively large, strongly acidic phenolic fraction. The authors considered the possibility that this fraction might have contained oestriol. Recently, HAGOPIAN AND LEVY[2] reported the isolation of radioactive oestriol after incubation of 16-^{14}C-oestradiol-17β with a coarse brei of fresh liver obtained from male adult rats. Shortly afterwards, BREUER, NOCKE AND KNUPPEN[3] found that oestradiol-17β when incubated with rat liver slices was metabolized not only to oestriol but also to 6-hydroxyoestradiol-17β and 6-hydroxyoestrone. However, owing to the lack of material none of metabolites could be isolated. It is the purpose of the present paper to describe a large-scale experiment which led to the isolation of 6-hydroxyoestradiol-17β and oestriol after incubation of oestradiol-17β with rat-liver slices.

EXPERIMENTAL

Male Sprague-Dawley rats, average weight 160–200 g, were killed by decapitation followed by exsanguination. The livers were removed as rapidly as possible and covered with isotonic ice-cold saline until sliced. Liver slices were obtained by the method of DEUTSCH[4].

The procedures used for the paper chromatography were as described by BREUER AND NOCKE[5].

Spectrophotometric measurements were made in a 1.0 cm cell with a Beckman spectrophotometer model DU.

The melting points were determined with a microscope hot-stage. The same thermometer was used for all determinations, and the values given are uncorrected for emergent stem (*cf.*[6]).

Reference steroids. 6-Hydroxyoestradiol-17β (m.p. 247–249°) was prepared by the reduction of 6-oxooestradiol-17β with sodium borohydride. In earlier papers[7–9] the hydroxyl group at position 6 was designated 6 "β" but recently WINTERSTEINER AND MOORE[10] published experiments on the basis of which 6-hydroxyoestradiol-17β is tentatively assigned the 6"α" (quasi-equatorial) configuration. 6-Hydroxyoestradiol-17β 3-methyl ether was prepared from 6-hydroxyoestradiol-17β by treatment with dimethyl sulfate in alkaline solution at 37°. The product was recrystallized from benzene–light petroleum and melted at 109–110°. 6-oxooestrone (246–248°) was obtained after oxidation of oestrone acetate with CrO_3 in acetic acid followed by hydrolysis of 6-oxooestrone acetate with 2 *N* methanolic KOH. The 3-methyl ethers of 6-oxooestrone (m.p. 146–147°) and of oestriol (m.p. 158–160°) were prepared from the respective free compounds by treatment with dimethyl sulfate. Acetylation of oestriol yielded oestriol triacetate (m.p. 128°).

RESULTS

Incubation

Incubations were carried out in 150 ml conical flasks containing 20 ml of Krebs phosphate saline[11] containing 20 m*M* glucose, pH 7.4, 6 g of liver slices and 0.4 ml ethanolic solution of oestradiol-17β (25 mg/ml). Thus, in the final reaction mixture the tissue–steroid ratio was 600 : 1. The flasks were not stoppered but were shaken vigorously during the incubation for 120 min at 37°. In this manner, a total of 600 mg of oestradiol-17β was incubated with approx. 360 g of liver slices.

Lipid extracts

After incubation the saline and the tissue were extracted twice with equal volumes of an ether–chloroform mixture (3 : 1, v/v) and once with an equal volume of ether. The combined extracts were washed twice with one-quarter volume of water, dried over sodium sulphate and evaporated to dryness. The yellow residue was dissolved in 100 ml of 70 % aqueous methanol; cholesterol and gummy impurities were removed by two washings with equal volumes of *n*-heptane. The aqueous methanol was then evaporated to dryness under reduced pressure. In order to separate the more "polar" material from the less "polar" one the semicrystalline residue was extracted 10 times with 100 ml portions of water. The aqueous extracts containing the more "polar" fraction were reextracted with equal volumes of benzene–light petroleum (1 : 1), and the combined aqueous extracts were then evaporated to 250 ml under reduced pressure. The aqueous phase was acidified with 15 ml of *N* HCl and thoroughly extracted with ether. The combined ether extracts (800 ml) were washed with 50 ml of 0.2 *M* $NaHCO_3$ and two 30 ml portions of water, dried over sodium sulphate and evaporated to dryness to yield the "polar" fraction (11 mg).

Methylation and chromatography

Previous experiments indicated that more than one substance was present in the "polar" fraction. An attempt to separate the single components from each other by chromatography on alumina failed, and it was therefore decided to attempt separation via the methyl ethers. The "polar" fraction was methylated with dimethyl sulphate as described by BROWN[12]. The methylated material was dissolved in benzene and then applied to an alumina column.

Investigation of fractions

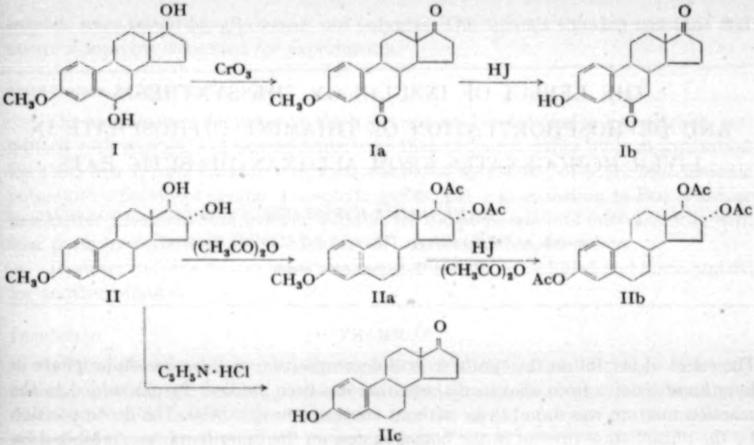
Fraction A. The fraction eluted with 20 ml of 1.4 % ethanol in benzene yielded 1.6 mg of colourless crystals (I), which were recrystallized from benzene–light petroleum and had m.p. 109°. After admixture with authentic 6-hydroxyoestradiol-17β 3-methyl ether the m.p. was not depressed. In 95 % ethanol solution I showed an u.v. absorption maximum at 280 mμ. For further identification, 1.3 mg of I were treated at room temperature for 3 h with a solution of CrO_3, 2.4 *N* with respect to oxygen, in 5 ml acetic acid. The excess of CrO_3 was destroyed with methanol, the solution diluted with water and then extracted with ether. 1.0 mg of colourless crystals (Ia) were obtained and recrystallized from light petroleum. Ia had a m.p. of 146° which was not depressed upon admixture with an authentic sample of 6-oxooestrone 3-methyl ether. The u.v. spectrum of Ia showed two maxima at 255 and 320 mμ and was identical with that of authentic 6-oxooestrone 3-methyl ether. Finally, Ia was demethylated using the method of HUFFMAN[13]. 1 ml of freshly distilled hydriodic acid was added to 0.5 mg of Ia dissolved in 1 ml of acetic acid. The mixture was heated for 5 min over a free flame, diluted after cooling with 20 ml cold aqueous sodium bisulphite and then extracted with ether. The material (Ib) obtained was subjected to paper chromatography in the system formamide–monochlorobenzene. After spraying the paper with FOLIN–CIOCALTEU reagent[14], a spot was detected which had the same R_F value as authentic 6-oxooestrone (0.23). The u.v. spectrum of Ib was identical with that of authentic 6-oxooestrone.

Fraction B. The fractions eluted with 30 ml of 3.0 % ethanol in benzene yielded 6.6 mg of colourless crystals (II) which gave a positive DAVID test[15]. After recrystallization from benzene the material melted at 158–160° and the mixed m.p. with oestriol 3-methyl ether was 158–159°. 3.3 mg of II were acetylated with acetic anhydride and pyridine; the diacetate (IIa) was demethylated by treatment with freshly distilled hydriodic acid as described by HUFFMAN[13]. After hydrolysis with methanolic KOH the material was again acetylated (IIb) and crystallized from methanol. After admixture with authentic oestriol triacetate (m.p. 128°) the m.p. was 127°. Another sample of II (3 mg) was converted to oestrone by heating it with 100 mg pyridine hydrochloride at 220° for 1.5 h[16]. The product obtained (IIc) was crystallized from ethanol and melted at 254–255°. After admixture with authentic oestrone the m.p. was not depressed. On paper chromatography in the system formamide–monochlorobenzene IIc showed the same R_F value as oestrone.

The reaction sequences establishing the identity of I with 6-hydroxyoestradiol-17β 3-methyl ether and of II with oestriol 3-methyl ether are shown in the reaction scheme.

DISCUSSION

The results reported here confirm and to some extent supplement the findings of HAGOPIAN AND LEVY[2] that oestradiol-17β can be transformed to oestriol in species other than man. The additional isolation of 6-hydroxyoestradiol-17β demonstrates that the hydroxylation of oestradiol-17β by rat liver slices is not confined to position 16 but also occurs at position 6 of the steroid molecule. It should be noted that 6-hydroxylation in the oestrogen series had previously been found with mouse liver preparations by MUELLER AND RUMNEY[7], and recently, 6-hydroxyoestrone has also been isolated from human urine by MARRIAN[17].



Reaction scheme.

ACKNOWLEDGEMENTS

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THE EFFECT OF INSULIN ON THE SYNTHESIS AND DEPHOSPHORYLATION OF THIAMINE DIPHOSPHATE IN LIVER HOMOGENATES FROM ALLOXAN DIABETIC RATS

KARL-HEINZ KIESSLING

Institute of Zoophysiology, University of Uppsala (Sweden)

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SUMMARY

The effect of insulin on the synthesis and decomposition of thiamine diphosphate in liver homogenates from alloxan diabetic rats has been studied. Insulin added to the reaction mixture was found to be without effect on the synthesis. The decomposition by the phosphatase present in the homogenates, on the other hand, was inhibited by added insulin.

The low content of thiamine diphosphate, previously found by others in liver homogenates from alloxan diabetic rats as compared with normal ones should therefore be caused by increased dephosphorylation of thiamine diphosphate rather than by impaired synthesis.

INTRODUCTION

A low content of thiamine diphosphate in alloxan diabetic rat livers compared with normal ones has been reported by several authors[1-3]. SILIPRANDI AND SILIPRANDI[2] attempted to explain the results as an impaired phosphorylation of thiamine. This explanation was supported by results obtained by FOA *et al.*[3] in experiments with homogenates from livers of alloxan diabetic rats. Addition of insulin caused the reduced TDP content to rise toward a normal level. These authors left open the possibility that the low concentration of TDP might be caused not only by an impaired synthesis but also by an increased dephosphorylation. Recently, VINCENT[4], with the aid of [^{35}S]thiamine, showed that no difference exists *in vivo* in the formation of radioactive TDP in the livers of normal and alloxan diabetic rats, and concluded that the phosphorylation of thiamine is not impaired in alloxan diabetes.

The present communication deals with the synthesis and dephosphorylation of TDP in liver homogenates from alloxan diabetic and normal rats in the presence and absence of insulin.

EXPERIMENTAL

Alloxan diabetes

Albino rats belonging to the Wistar stock, weighing between 150 and 250 g were used. 200 mg/kg alloxan were given intraperitoneally, and after one week the

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Abbreviations: TDP, thiamine diphosphate, ADP, adenosine diphosphate, ATP, adenosine triphosphate, TCA trichloroacetic acid.

animals were tested for glycosuria and polyuria. The animals showing constant and severe symptoms were used for experiments.

Preparation of liver homogenates

The animals were decapitated, the livers removed and chilled in ice, weighed, and minced with scissors. 1:4 homogenates were then prepared either by homogenization for 2 min in a Waring blendor at 0° with four parts, by volume, of an ice-cold, isotonic potassium chloride–potassium phosphate buffer, pH 7.5, according to **Foa** *et al.*[3], or in a Potter-Elvehjem homogenizer, suitable for the preparation of mitochondria, with four parts by volume of an ice-cold 0.25 *M* sucrose solution for 2 min.

In either case the tissues were homogenized for 1 min, rechilled and homogenized for another minute.

Incubation

1 ml of the homogenate was transferred to a reaction vessel together with magnesium, creatine, glutamate, potassium citrate, cytochrome *c* and ADP mainly as described by **Foa** *et al.*[3]. When phosphate was not already present in the medium a potassium phosphate buffer, pH 7.5 was added.

When the synthesis of TDP from thiamine was to be studied, 50 μg of thiamine, radioactive inorganic phosphate, and 50 μg of synthetic nonradioactive TDP were added. When inorganic non-radioactive phosphate was not already present, it was added to a concentration of 0.1 m*M*. Immediately before the incubation was started 0.8 units of insulin were added to some vessels. The purpose of adding non-radioactive TDP was to dilute the TDP being synthesized during the incubation and to render it less accessible to phosphatases. At the end of the incubation the reaction was stopped by addition of TCA to a final concentration of 8 %, and the precipitated proteins removed by centrifugation. TCA was removed with ether.

To the reaction mixtures in which the dephosphorylation of TDP was to be determined the additions were the same as described above, only that synthetic radioactive TDP, prepared according to **Kiessling**[5], was added instead of thiamine and radioactive inorganic phosphate. However, the concentration of non-radioactive inorganic phosphate was increased to 5 m*M*. This was done in order to dilute the radioactive phosphate liberated from [^{32}P]TDP during the incubation, thus allowing only a negligable amount to be reincorporated into organic compounds. The reactions were terminated by adding 5 *M* H_2SO_4 to a final concentration of 0.5 *M*, and the precipitated proteins removed by centrifugation.

The insulin used was one of two types. The one type, an amorphous insulin*, was reprecipitated 3 times with ethanol–aceton for the removal of zinc which might interfere. The other type was a zinc-free commercial insulin from Vitrum.

Isolation of TDP and measurement of radioactivity

Of the samples in which the synthesis of TDP was to be studied 0.5 ml were added as a line on a Whatman No. 1 paper chromatogram, and developed according to **Siliprandi and Siliprandi**[6]. The TDP band was extracted from the chromatogram, and purified twice by another method described by **Kiessling**[7], using Whatman 52 filter paper washed according to **Eggleston and Hems**[8]. The radioactivity of TDP was then determined after wet ashing, measurement of phosphorus in a Beckman spectrophotometer, and counting of the radioactivity in an M 6 liquid counter tube. The radioactivity of the TDP synthesized in the whole sample has been calculated as % of the total radioactivity of the sample.

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* Kindly supplied by Professor E. **Jorpes**.

When studying the amount of TDP decomposed by phosphatases 1.0 ml of the samples were extracted with benzene–isobutanol according to ERNSTER *et al.*[9], and the radioactivity of the two phases measured. This allows a calculation of the percentage hydrolysed of the TDP added. In either type of the incubations described a control was made to which TCA and H_2SO_4, respectively, were added before the homogenates. They were then treated in the same way as the samples incubated, and deducted from the incubation values.

RESULTS

The effect of insulin on the synthesis of TDP in liver homogenates from diabetic rats

The homogenate was prepared in a Potter-Elvehjem homogenizer, and insulin added immediately before the incubation was started. The results from five different rats made diabetic by alloxan injections are seen in Table I.

The figures in Table I show no significant difference ($p > 0.4$) in the ability of the liver homogenate to synthesize TDP from thiamine in the presence or absence of insulin. In some experiments insulin was also added after certain intervals throughout the incubation for the compensation of a possible destruction by insulinase, but the results were the same as with insulin given as a single dose at the beginning of the incubation.

The effect of insulin on the dephosphorylation of TDP in liver homogenates from diabetic rats

In a homogenate prepared with the Waring blendor added TDP was rapidly dephosphorylated, while with use of the Potter-Elvehjem homogenizer the dephosphorylation was slow. The reason for this probably is that the TDP splitting phosphatases are enclosed in the lysosomes which according to GIANETTO AND DE DUVE[10] contain hydrolyzing enzymes, which are set free by homogenizing the liver in a blendor; using a Potter-Elvehjem homogenizer this should not occur to the same extent.

The first experiments on the effect of insulin were performed with homogenates prepared in a blendor. Fig. 1. shows the results from a typical experiment. Already after an incubation of 5 min at 27° only 30% of the added TDP was left in the presence of 1 ml 1:4 liver homogenates from a diabetic rat. Addition of insulin before incubation partly inhibited TDP break-down.

Also in the presence of homogenates of livers from normal rats, prepared in the same way, insulin decreased the rate of TDP decomposition. Fig. 2, giving the results of a typical experiment, also shows that zinc ions are without effect on the decomposition rate. Thus the insulin effect is not caused by traces of Zn, eventually present in the preparation used.

It being impossible to reproduce the experiments exactly since the TDP splitting activity of the homogenates of rat livers differed very widely, a very big material would be needed for establishing an eventual significant difference between homogenates of livers from normal and diabetic rats. From the assembled data it could only be concluded that there seems to be a tendency towards a more rapid break-down of TDP in homogenates from alloxan diabetic rat livers than from normal ones.

When the homogenates have been prepared in a Potter-Elvehjem homogenizer, the break-down of TDP is very slow. In the presence of 1 ml 1:4 liver homogenates

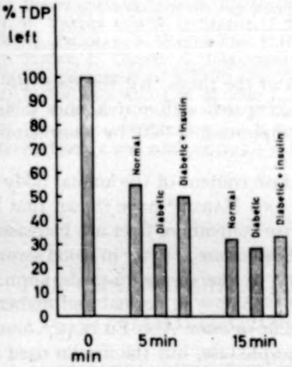

Fig. 1. The effect of insulin on the dephosphorylation of TDP in liver homogenates from normal and alloxan diabetic rats. The piles indicate % TDP remaining after incubation for 0, 5 and 15 min at 27°. For reaction mixtures see EXPERIMENTAL. The normal and the diabetic rats used in the experiment represented above were twins of the same sex, and apart from the injection of alloxan, treated in exactly the same way. The homogenates were prepared by mincing the tissues in a high speed Waring blendor. Both types of insulin, described in EXPERIMENTAL, were tested in parallel experiments, and gave the same effect.

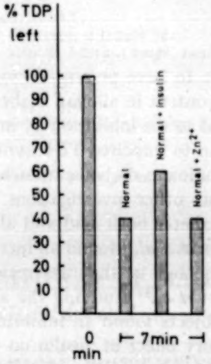

Fig. 2. The effect of insulin and of zinc ions on the decomposition of TDP in liver homogenates from normal rat. The piles indicate % TDP remaining after incubation for 0 and 7 min at 27°. For reaction mixtures see EXPERIMENTAL. The homogenate was prepared in a high speed Waring blendor. To the fourth vessel 1 mmole $ZnCl_2$ was added.

TABLE I

TDP SYNTHESIZED IN LIVER HOMOGENATES FROM ALLOXAN DIABETIC RATS

The tissue was homogenized in a Potter-Elvehjem homogenizer. The incubation was performed at 37° for 60 min. The figures show how much of the radioactivity was found in the whole sample as TDP-phosphorus in % of the total radioactivity of the sample. For reaction mixtures see EXPERIMENTAL. $t = 0.266$, $p > 0.4$.

Expt.	*TDP synthesized*	
	Insulin	*No insulin*
1	$1.66 \cdot 10^{-2}$	$1.42 \cdot 10^{-2}$
2	$1.20 \cdot 10^{-2}$	$1.42 \cdot 10^{-2}$
3	$0.86 \cdot 10^{-2}$	$0.86 \cdot 10^{-2}$
4	$0.75 \cdot 10^{-2}$	$0.84 \cdot 10^{-2}$
5	$0.99 \cdot 10^{-2}$	$1.02 \cdot 10^{-2}$

TABLE II

THE EFFECT OF INSULIN ON THE DEPHOSPHORYLATION OF TDP IN LIVER HOMOGENATES FROM ALLOXAN DIABETIC RATS

The figures are % of TDP decomposed by 0.2 g of liver during an incubation of 60 min at 37°. The homogenates had been prepared in a Potter-Elvehjem homogenizer. For reaction mixtures see EXPERIMENTAL. $t = 7.50$, $p < 0.0005$.

Expt.	*% TDP decomposed*	
	Insulin	*No insulin*
1	4.4	8.2
2	3.6	7.8
3	2.0	5.0
4	1.2	3.9
5	4.8	10.8
6	5.6	11.3

from diabetic animals, at most 12% of the added TDP is hydrolyzed during 60 min at 37°. In Table II the results from six experiments are found. They show that the inhibiting effect of insulin on the hydrolytic effect of liver homogenates from diabetic animals is very significant ($p < 0.0005$). When insulin has been added, on the average the decomposition of TDP is reduced by about 50%.

DISCUSSION

We believe to have presented evidence in support of the thesis that the decrease of the TDP content in alloxan diabetic rat livers as compared with normal ones should be ascribed to the inhibition by insulin of the decomposition of TDP by phosphatase, rather than to impaired TDP synthesis.

That alloxan diabetes influences the phosphatase content of the animal body is known from other investigations. Thus DRABKIN AND MARSH[15] have shown that in alloxan diabetes both acid and alcaline phosphatase contents of liver are increased, while CANTOR *et al.*[16] found an increased alcaline phosphatase activity in blood serum. Insulin injected in the rats restored the activity of the serum towards normal. DIOGUARDI *et al.*[17] studying the alcaline serum phosphatase in normal and diabetic human subjects found an inhibitory effect of insulin *in vitro*. Also FILTEAU[18] found an inhibitory effect of insulin on the glucose-6-phosphatase, but the insulin used in his *in vitro* experiments containing zinc, he could not definitely exclude the effect to be due to zinc rather than to insulin.

The evidences presented above that an increased decomposition rather than an impaired synthesis should be the cause of the lower TDP content in alloxan diabetic animals as compared with normal are based upon studies on liver homogenates from diabetic rats in the presence and absence of insulin. Although the results obtained with a normal rat-liver homogenate are presented in Fig. 1, the experiments done on this material are too few to allow definite conclusions about an increased decomposition of TDP in diabetic animals as compared with normal. As said already, in both kinds of homogenates the deviations of the individual values from the means were so great that only a very great number of experiments could have given convincing evidence. The great variations within the groups have probably been caused by lack of control of the damage done to the cell components in particular the lysosomes and nuclei, in both procedures used for preparing the homogenates.

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THE CHARACTERIZATION OF THE PATHWAY OF MALTOSE UTILIZATION BY *ESCHERICHIA COLI*

I. PURIFICATION AND PHYSICAL CHEMICAL PROPERTIES OF THE ENZYME AMYLOMALTASE

HERBERT WIESMEYER*, ** AND MELVIN COHN***

Department of Microbiology, Washington University School of Medicine, St. Louis, Mo. (U.S.A.)

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SUMMARY

A method for the purification of amylomaltase, an inducible enzyme of *E. coli*, has been presented. The enzyme sediments as a single symmetrical peak and was found to have a molecular weight of 124,000. The absorption spectrum, solubility and immunochemical properties of the enzyme are described.

INTRODUCTION

The utilization of maltose by *Escherichia coli* involves two distinct events; the entrance of the sugar into the cell *via* a specific concentrating system, termed the maltose

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The following abbreviations will be used: TPN, triphosphopyridine nucleotide (oxidized form); TPNH, triphosphopyridine nucleotide (reduced form); ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane.

[*] This work is a part of the thesis submitted by H. Wiesmeyer to the Graduate School of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[**] Present address: School of Biology, Johns Hopkins University, Baltimore, Maryland.

[***] Present address: Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California.

permease[1], and its subsequent metabolism initiated by the enzyme amylomaltase.

Amylomaltase previously described by MONOD AND TORRIANI[2] and DOUDOROFF *et al.*[3] is extractable from maltose grown cells and catalyzes the reversible conversion of maltose to glucose and a polymer consisting of α-1,4-glucosidic units. The reaction is as follows:

$$\text{n maltose} \rightleftharpoons \text{n glucose} + (\text{glucose})\text{n}$$

The size of the polymer, (glucose)n, is influenced by the concentration of free glucose[3]. Under equilibrium conditions the polymer is of the order of 4–6 glucose units. Under conditions where the liberated glucose is not permitted to accumulate the polymer gives the typical starch-like response with iodine[2]. The absence of a phosphate requirement would place this enzyme in the category of the transglucosidases proposed by DOUDOROFF *et al.*[4], in which an enzyme–glucose complex is presumably formed and retains the energy originally present in the glycosidic linkage.

This investigation will be described in three papers; the first will deal with purification and the physical properties of amylomaltase, the second with its enzymic properties, and the third with the system involved in the uptake and concentration of maltose within the bacterial cell.

METHODS AND MATERIALS

Preparation of Escherichia coli *for the isolation of amylomaltase*

E. coli ML 308 was grown in an inorganic salts medium, M56, the composition of which is listed below. The medium was supplemented with 0.4 % maltose, autoclaved separately. $MgCl_2 \cdot 6H_2O$, $1 \cdot 10^{-3}$ *M*; $(NH_4)_2SO_4$, $1.5 \cdot 10^{-2}$ *M*; $FeSO_4 \cdot 7H_2O$, $2 \cdot 10^{-6}$ *M*; Potassium phosphate buffer, pH 7, $1 \cdot 10^{-1}$ *M*.

50–100 l of maltose limited culture grown at 37° were harvested by passage through the Sharples centrifuge. The yield was of the order of 4 g wet wt./l. The cells were then washed twice with volumes of cold distilled water equal to their packed weight, recentrifuged and frozen as a paste at —20° until needed. The frozen cells retain full enzyme activity for at least one year.

Assay procedure

The cleavage of maltose by amylomaltase results in the liberation of free glucose. The rate of glucose formation was determined by its conversion to glucose-6-phosphate and then to 6-phosphogluconic acid by the sequential action of hexokinase and glucose-6-phosphate dehydrogenase. The latter enzyme oxidizes the glucose-6-phosphate to 6-phosphogluconic acid with the concommitant reduction of TPN. This reduction can be followed spectrophotometrically. The extinction coefficient of TPNH is $6.23 \cdot 10^3$ cm^{-1}/mole[5] as determined at 340 mμ.

The assay mixtures for the determination of amylomaltase activity consisted of 4 μmoles potassium phosphate buffer pH 6.9, 1 μmole $MgCl_2$, 0.5 μmole ATP, 0.2 μmole TPN, 2 μmoles maltose, and an amount of hexokinase and glucose-6-phosphate dehydrogenase activity 10 times greater than that of the amylomaltase being assayed. The total volume of the reaction mixture was 1 ml. The assays were performed at 28°, and the O.D. was measured using either the Beckman DU spectrophotometer or the Cary automatic recording spectrophotometer.

A unit of amylomaltase activity is defined as that amount of enzyme which will liberate 1 mμmole of glucose per min at 28°, pH 6.9. Specific activity is defined as units/mg protein. Protein concentrations were determined by the method of LOWRY *et al.*[6].

With the above assay system, the rate of glucose formation is directly proportional to the concentration of amylomaltase being 1.2, 2.4 and 5.0 mμmoles/min at a concentration of 42, 84 and 168 μg protein/ml of crude extract.

Enzyme preparations

Hexokinase and glucose-6-phosphate dehydrogenase: The source of the hexokinase and glucose-6-phosphate dehydrogenase was a partially purified extract prepared from glucose grown *E. coli* B shown to be free of maltase and TPNH oxidase. The procedure for the purification of both the hexokinase and glucose-6-phosphate dehydrogenase is described below:

E. coli strain B, grown in synthetic medium 56 supplemented with glucose (4 g/l), was harvested by centrifugation and washed with 50 volumes of cold (0–4°) distilled water. The washed cells were resuspended in 0.01 *M* Tris buffer, pH 7.5 at a ratio of 1 part cells to 4 parts buffer. The suspension was sonicated for 20 min in the Raytheon 10 kc sonic oscillator and the cellular debris removed by centrifugation. The resulting supernatant fluid, after dilution with an equal volume of distilled water, was treated with streptomycin sulfate to a final concentration of 0.2 %. The resulting streptomycin precipitate was removed by centrifugation and discarded and the supernatant was treated with ammonium sulfate to 38 % of saturation. A precipitate formed and was discarded. This latter ammonium sulfate supernatant was further treated with ammonium sulfate to 55 % of saturation and the 38–55 % ammonium sulfate precipitable material was collected by centrifugation and redissolved in 0.05 *M* potassium phosphate buffer pH 7.0. This solution was then centrifuged for 1 h at 30,000 rev./min in the Spinco Model L preparative centrifuge. The supernatant of this last centrifugation contained both the hexokinase and glucose-6-phosphate dehydrogenase enzymes.

The purified hexokinase-glucose-6-phosphate dehydrogenase preparation has a specific activity which is 30 times greater than the sonic extract and represents 70 % of the original starting activity. This material can be lyophilized and stored at −15° for at least six months without any appreciable loss in activity. When needed, the dry powder is dissolved in cold 0.01 *M* potassium phosphate buffer, pH 7.0.

Purification of amylomaltase: The source of the enzyme amylomaltase was *E. coli* strain ML 308. Strain ML 308 can be induced by maltose to produce amylomaltase following a kinetics shown in Fig. 1. The formation of enzyme is a linear function of increase in bacterial mass. This finding has been discussed previously[7,8]. The slope of this curve calculated using the finding that 1 unit enzyme = 0.106 μg protein (see later), shows that 1.8 % of the total protein being synthesized is specifically amylomaltase under these conditions of induction.

Unfortunately the large scale preparation of bacteria for the purification of this enzyme was carried out before the optimal conditions for amylomaltase synthesis were known so that the isolation procedure described here was made, starting with induced cells at 10 % of the above level of activity.

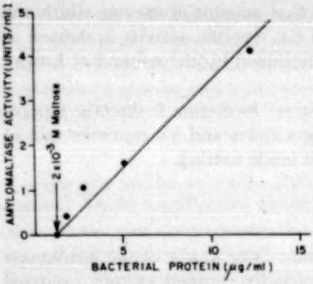

Fig. 1. At the point indicated, $2 \cdot 10^{-3}$ *M* maltose was added to a culture of *E. coli* ML 308 growing exponentially in M56 supplemented with $5 \cdot 10^{-3}$ *M* succinate. 5-ml aliquots of the culture were removed at various times and centrifuged. The cells were resuspended in fresh M56 + succinate containing 10 μg desoxycholate and 1 drop of toluene/ml. The suspension was shaken at 37° for 30 min, following which the cells were removed from suspension by centrifugation. This treatment has been shown in the case of amylomaltase to reveal the maximum enzyme activity by destroying the permeability barrier (discussed in 8). After removal of the supernatant fluid, the cells were again resuspended in M56 + maltose (5 μmoles/ml) and incubated at 37° for 30 min. Following this incubation the reaction mixture was boiled for 3 min and glucose determined in the supernatant fluid by the hexokinase-glucose-6-phosphate dehydrogenase assay system.

The procedure for the growth of this strain and the medium used has been described above. The purification of the amylomaltase of ML 308 follows.

E. coli ML 308 was suspended in distilled water at a ratio of 1 part cells to 5 parts water at 0–4°. 60-ml aliquots of this suspension were sonicated for 20 min in a Raytheon 10 kc oscillator. The sonicates were centrifuged for 1 h at 30,000 rev./min (78,410 × *g*) in the Spinco Model L preparative centrifuge. The sediment of this centrifugation was discarded and a 20% solution of streptomycin sulfate (Merck and Co.) to a final concentration of 5% was added to the supernatant. The resulting precipitate was removed by centrifugation and discarded. Saturated ammonium sulfate (710 g/l) was added to the supernatant to a final concentration of 45% of saturation. The resulting precipitate was discarded and ammonium sulfate to a final concentration of 55% saturation was again added. The 45–55% ammonium sulfate fraction was redissolved in 0.1 *M* potassium phosphate buffer pH 7.0. This material was then adsorbed onto a titrated minimum amount of alumina C γ gel and the gel–enzyme complex washed with water, 0.1 *M* phosphate buffer and eluted with 0.33 *M* phosphate buffer pH 7.0. The alumina C γ eluate was dialyzed overnight against 0.1 *M* phosphate buffer pH 7.0. The dialyzed eluate was then adsorbed onto calcium phosphate gel and after washing the gel–enzyme complex with water and 0.1 *M* phosphate buffer pH 7.0, the enzyme was eluted by washing with 0.33 *M* phosphate buffer pH 7.0. The calcium phosphate eluate was approximately 500 times as pure as the original crude extract. The data for this purification procedure are in Table I.

TABLE I

THE PURIFICATION OF AMYLOMALTASE

Fraction	*Total enzyme*	*Total volume*	*Specific activity*	*Per cent recovery*
	Units × 10^{-5}	*(ml)*	*Units/mg protein*	
Crude sonicate	21	4200	18	100
Streptomycin supernatant	24	6100	56	114
45–55% of saturation of ammonium sulfate (precipitate)	16.8	800	312	80
Alumina C γ gel eluate	8	935	3840	38
Calcium phosphate gel eluate	6.3	500	9400	30

Immunization of rabbits

The purified amylomaltase was used for the immunization of two rabbits. 24-ml of a 0.1 % protein solution was treated at 0° with alum at a ratio of 1 part of protein to 2.7 parts of alum. The preparation was adjusted to pH 6.2 with 0.1 *N* sodium hydroxide. The enzyme was absorbed onto the gel-like precipitate which formed.

The enzyme–gel complex was injected intravenously into each rabbit 4 times weekly for a period of four weeks at which time a total of 12 mg of purified amylomaltase protein had been given. The amount of protein which was injected each time was gradually increased from 0.1 mg at the beginning to 2 mg at the end. On the fifth week the animals were bled.

The antiserum was heated at 56° for 30 min to destroy the maltase normally present.

EXPERIMENTAL

Physical studies on the purified enzyme

The absorption spectrum of amylomaltase: The absorption spectrum of the purified amylomaltase is shown in Fig. 2. The absorption spectrum of amylomaltase examined between 2000 and 4000 Å. showed an inflection point of 2900 Å with a 2800/2900 ratio of 1.29, and a rather low 2800/2600 ratio of 1.32. The purified protein has a maximum absorption at 2780 ± 20 Å.

The extinction coefficient at 2780 Å is 3.7 cm^2 mg^{-1}.

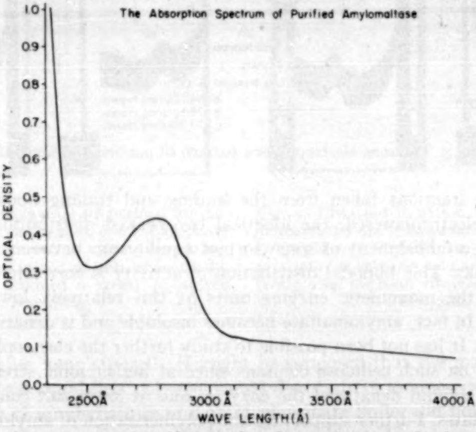

Fig. 2. The absorption spectrum of purified amylomaltase was prepared using 120 μg protein/ml in 0.1 *M* potassium phosphate buffer pH 7.0. Tracing was obtained using the Cary automatic recording spectrophotometer.

The electrophoretic pattern of amylomaltase: The procedure described by PORATH[9] for the electrophoresis of proteins on cellulose columns was employed for the investigation of the electrophoretic properties of amylomaltase.

The calcium-phosphate gel eluate was dialyzed against 0.066 *M* phosphate buffer pH 7.0 and 2 ml of this preparation containing 7.2 mg protein/ml were placed on a cellulose colum 1 cm in diameter and 50 cm in length. The column had previously been washed with the buffer against which the enzyme preparation had been dialyzed. A potential of 700 V and a current of 25 mA was applied to the column for a period of 20 h. The column was cooled by a jacket in which ice water circulated. The enzyme was eluted with the above buffer and 1.2-ml fractions were collected. The absorption of the aliquots at 260 and 280 mμ as well as the enzymic activity was determined. The elution pattern of a typical experiment is shown in Fig. 3. The more negatively charged leading peak (A) has about the same specific activity as the dialyzed starting material. However, the slower moving component (C) has a significantly higher specific activity. The activity of the dialyzed starting material was somewhat lower than the best preparation described in Table I. This loss of activity occurs at the ionic strength used for electrophoresis.

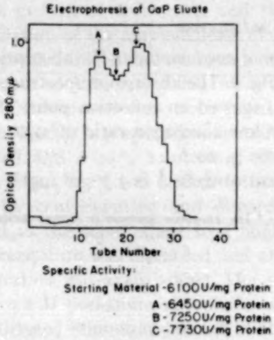

Fig. 3. The zone electrophoresis pattern of purified amylomaltase.

When the fractions taken from the leading and trailing boundaries were redialyzed and electrophoresed, the identical two-peaked distribution resulted. This suggested a re-establishment of some sort of equilibrium between the components of the two peaks. This bimodal distribution of activity is correlated with the polymerization of the monomeric enzyme units at this relatively low phosphate ion concentration. In fact, amylomaltase becomes insoluble and is denatured at very low ionic strengths. It has not been possible to study further the electrophoretic behavior of the enzyme on such cellulose columns since at higher ionic strengths, excessive heating of the column denatured the enzyme, and at lower salt concentrations, the protein precipitates. Further support for the polymerization of amylomaltase is given by the ultracentrifuge studies.

Sedimentation of amylomaltase in the ultracentrifuge: The ultracentrifuge studies on the purified amylomaltase were performed using the Spinco Model E ultracentrifuge. Of particular interest was the effect of high and low phosphate ion concentration on the sedimentation pattern of the purified protein. When the purified enzyme was dialyzed against 0.066 *M* phosphate buffer pH 7.15 and centrifuged at 59,780 rev./min, two peaks appeared when viewed with the schlieren optics. The minor, faster moving component appeared 20 min after the rotor had attained maximum speed, and separated from the major component some 48 min later. Fig. 4 shows representative pictures taken during the run.

One interpretation of the finding that amylomaltase precipitates from solution at phosphate buffer concentrations of less than 0.1 *M* was that the enzyme polymerized. This hypothesis led to the suggestion that the faster moving component was a polymeric form of the enzyme which preceded the stage leading to precipitation. Based on this assumption, the same enzyme preparation which showed two components was redialyzed against 0.14 *M* phosphate buffer and recentrifuged. The results of this treatment are shown in Fig. 5. At this higher ionic strength the faster moving component was no longer detectable, thus supporting the hypothesis of a reversible equilibrium between the two forms of the enzyme.

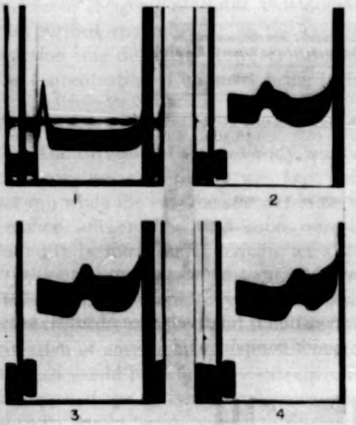

Fig. 4. Ultracentrifugation of purified amylomaltase after dialysis against 0.066 *M* phosphate buffer pH 7.15. Pictures 1, 2, 3 and 4 were taken 4 min, 56 min, 72 min and 92 min, respectively, after the rotor attained a speed of 59,780 rev./min.

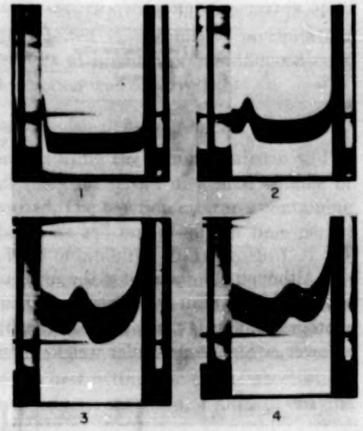

Fig. 5. Ultracentrifugation of purified amylomaltase after dialysis against 0.14 *M* phosphate buffer pH 7.15. Pictures 1, 2, 3 and 4 were taken at 4 min, 24 min, 60 min and 92 min, respectively, after the rotor attained a speed of 59,780 rev./min.

At still higher ionic strength, it might be supposed that the material described above, which itself could be a polymer, would dissociate to monomeric units. Analysis of the behavior of amylomaltase in 0.25 *M* phosphate buffer did not reveal the presence of any component of lower sedimentation constant. It was assumed, therefore, that under these conditions, the enzyme was in its monomeric form.

The determination of the molecular weight of amylomaltase: The procedure used for the determination of molecular weights was that proposed by ARCHIBALD[10] and developed by KLAINER AND KEGELES[11]. The molecular weight of the amylomaltase in 0.1 *M* potassium phosphate buffer pH 7.2, was calculated from the data derived from two separate ultracentrifuge runs. The first was essentially a sedimentation equilibrium run in which pictures were taken at short intervals for a period of 1–2 h. The second run was performed in a synthetic boundary cell and served to determine the relative concentration of material used for the study. The pictures in both experiments were taken at an angle of 75°. The speed of the rotor for the sedimentation equilibrium run was 10,589 rev./min, and the temperature of the rotor was 25°.

Eight pictures were taken in the sedimentation equilibrium experiment each at 8 min intervals. The molecular weight was calculated from the concentration gradients at the top and bottom of the cell. This value was calculated from the pictures taken 16, 24, 32 and 48 min after the rotor had reached the desired speed. The values obtained for the molecular weight and the pictures from which they were determined are shown in Table II and Fig. 6. The average mol. wt. was found to be 124,000.

TABLE II

THE MOLECULAR WEIGHT OF AMYLOMALTASE DETERMINED BY THE METHOD OF ARCHIBALD[10]

Time of picture after rotor reached speed (min)	*Molecular weight calculated from the concentration gradient established at the top and bottom of the cell*	
	Top	*Bottom*
16	124,300	116,000
24	139,500	138,300
32	119,300	120,000
48	115,700	—
	Average = 124,000	

Although the values for the molecular weight vary somewhat from one picture to another (less than 15 % between extremes), they agree closely within any particular photograph. This is taken to mean that the preparation is relatively free of substances of lower or higher molecular weight than the major component.

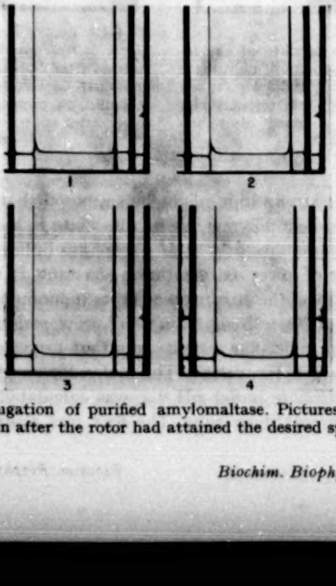

Fig. 6. Low speed centrifugation of purified amylomaltase. Pictures 1, 2, 3 and 4 were taken at 16, 24, 32 and 48 min after the rotor had attained the desired speed of 10,589 rev./min.

A second point of interest is the width of the oil–solution interphase. The width of this sharp interphase remained constant throughout the experiment, indicating the absence of any aggregates which might have formed during the course of the run. The presence of such substances would have been revealed by a thickening of this boundary.

The immunological characterization of amylomaltase

Immunological homogeneity: When studied by simple diffusion in agar[12] the antiserum to the purified amylomaltase gave a single zone of precipitation with up to 1 mg/ml of purified amylomaltase. However with crude extracts from induced *E. coli* two zones were seen, one of them rather faint. The second faint zone of precipitation is also present when extracts of non-induced cells are used. Since the rabbits were immunized with the purified enzyme, there must be present in it trace amounts of unrelated antigenic material. This unrelated material is present in such low level in the purified enzyme that at the concentration tested (1 mg/ml) no precipitation reaction was detected. However in crude extracts of induced or non-induced cells the concentration of impurity being higher, a reaction was observed.

The reaction of inhibition of activity between anti-amylomaltase and the enzyme

The titration of the antibody was carried out using the serum diluted 1:5. The concentration of antibody was kept constant (0.05 ml serum in a final volume of 0.2 ml) while the enzyme concentration was varied. The reaction mixtures containing enzyme, anti-enzyme, and saline were incubated at 28° for 30 min, a time period found to be more than adequate for a stable level of inhibition to be reached.

Controls using normal serum, taken prior to immunization, and saline were also included. Following the 30 min incubation, aliquots from the various incubation mixtures were removed and then enzyme activities determined. Fig. 7 illustrates the titration of amylomaltase by anti-enzyme. A unit of amylomaltase considered as an antigen would be defined by extrapolation of the best fitting line to the abscissa. In this case, if the antiserum concentration was taken as a standard, 4 units of enzyme activity would be equivalent to 1 antigenic unit.

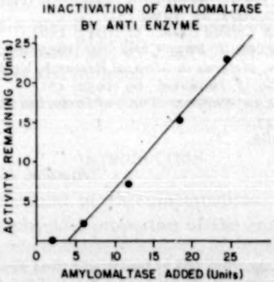

Fig. 7. Stoichiometry of the inactivation of amylomaltase by rabbit anti-enzyme.

The search for proteins immunologically related to amylomaltase: There are two examples of serologically related protein systems present in a given clone of bacteria, the PZ-β-galactosidase[13] and the tryptophane synthetase-CRM proteins[14]. Furthermore, as is already known in the galactosidase system[15], certain mutants unable to utilize maltose might possess inactive altered enzyme protein recognizable as an antigen by the anti-amylomaltase serum.

Using precipitation absorption methods similar to those used in the PZ-β-galactosidase system a search was made for cross reacting proteins using extracts from induced and non-induced cells. No proteins of the PZ-type described for the β-galactosidase system were detectable.

The effort to detect related proteins was extended using the reaction of inhibition described above. The titration end point of amylomaltase was always the same in extracts of induced cells, or in extracts of non-induced cells or various maltose negative mutants to which purified enzyme was added. If CRM proteins of affinity for anti-amylomaltase similar to that of the enzyme were present, they would have been detected.

ACKNOWLEDGEMENTS

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THE CHARACTERIZATION OF THE PATHWAY OF MALTOSE UTILIZATION BY *ESCHERICHIA COLI*

II. GENERAL PROPERTIES AND MECHANISM OF ACTION OF AMYLOMALTASE

HERBERT WIESMEYER*, ** AND MELVIN COHN***

Department of Microbiology, Washington University School of Medicine, St. Louis, Mo. (U.S.A.)

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SUMMARY

The enzymic behavior of the purified amylomaltase was investigated and the following observations reported:

1. The enzyme has a pH optimum of 6.9.

2. The turnover number of amylomaltase was found to be 1160 moles of maltose/mole of enzyme/min at 28° and pH 6.9.

3. The enzyme acts upon maltose with the release of one molecule of glucose for each maltose molecule cleaved.

4. The reaction catalyzed by amylomaltase has an equilibrium constant close to one (depending on the size of the polymer formed).

5. Heavy metals, such as Hg^{++}, Cu^{++}, Ni^{++} and Zn^{++}, inhibit the enzyme.

6. The mechanism of action of amylomaltase indicates the release of the reducing end of the maltose molecule as free glucose, whereas the non-reducing end is contributed toward the formation of the polymer.

7. Studies pertaining to the substrates and inhibitors of the enzyme indicated the requirement for equatorial hydroxyl groups at carbon atoms 2 and 4 of the non-reducing end of the molecule, and the presence of carbon 6 of both the reducing and non-reducing ends of the molecule and the requirement of an unsubstituted carbon atom 1 of the reducing end of the molecule.

INTRODUCTION

A procedure for the purification of the amylomaltase of *Escherichia coli*, and a description of some of the physical properties of the enzyme were presented in the

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The abbreviations used here are to be found in the previous paper[1].

[*] This work is a part of the thesis submitted by H. WIESMEYER to the Graduate School of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[**] Present address, School of Biology, Johns Hopkins University, Baltimore, Maryland.

[***] Present address, Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California.

preceding paper[1]. The purpose of the present report is to describe in further detail[2,3] the enzymic properties of amylomaltase.

METHODS AND MATERIALS

Chemical preparations

All carbohydrates used in this investigation, with the exception of β-methyl maltoside, amylose and the radioactive compounds, maltose and maltobionic acid, were commercially available. The maltose* used for the assays was recrystallized four times from 80 % ethanol in order to reduce its glucose content to less than 0.05 %. The other sugars used in the substrate studies were not recrystallized. β-methyl maltoside was prepared according to the procedure of D. H. BRAUNS[4] and SCHOCH, WILSON AND HUDSON[5]. The preparation of amylose and specifically labeled maltose and maltobionic acid is described below.

Preparation of amylose: MONOD AND TORRIANI[2] demonstrated the formation of a starch-like polymer under conditions where the glucose released by the action of amylomaltase on maltose was not allowed to accumulate. The procedure employed by these investigators was the oxidation of glucose to gluconic acid by notatin[2]. The reaction was run in a Warburg apparatus and followed by the uptake of molecular oxygen.

The method used in the present studies for the removal of glucose involved the conversion of this sugar to glucose-6-phosphate by the addition of ATP and hexokinase to the reaction mixture. The formation of glucose-6-phosphate was followed either by (a) continuous automatic titration to pH 7.0 of the acid produced in a weakly buffered medium and resulting from the phosphorylation of the hexose, or (b) spectrophotometrically by the conversion of the phosphate ester to 6-phosphogluconic acid by means of the glucose-6-phosphate dehydrogenase-TPN system.

A typical reaction mixture contained 5 μmoles maltose/ml, 2 μmoles $MgCl_2$/ml, 5 μmoles ATP/ml, hexokinase and amylomaltase. The pH was maintained at 7.0 by the addition of 0.01 *N* KOH. Following the completion of the reaction, the incubation mixture was made alkaline to brom thymol blue and passed through a Dowex-1-chloride anion exchange resin. The effluent was collected in absolute ethanol. The resulting precipitate was removed from solution by centrifugation and redissolved in cold distilled water. The solution was again treated with ethanol and the material precipitable in 50 % alcohol collected by centrifugation and dried over solid KOH under vacuum. This material is water soluble (although it steadily loses this property over several weeks), precipitable in 50 % ethanol and reacts with iodine to give a typical blue color characteristic of a starch reaction.

It has been found necessary to maintain the maltose concentration at or below 5 μmoles/ml in order to obtain long chain polymers. Presumably at higher levels of maltose, the concentration of glucose acceptors is such that the average chain length of the polymer formed is lower than that required to give the typical iodine reaction or be precipitable in 50 % ethanol.

It has been possible to obtain yields of 30–80 % of theory, assuming that only one glucose unit of the maltose molecule goes toward the formation of the polymer.

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[*] The maltose obtained from Pfanstiehl Laboratories, Inc., Waukegan, Ill., contained 3–5 % glucose.

Preparation of specifically labeled maltose: Specifically labeled maltose was prepared from [^{12}C]amylose and [^{14}C]glucose by taking advantage of the fact that the reaction catalyzed by amylomaltase was reversible. As will be shown in the experimental section, the maltose thus prepared is labeled in the reducing end of the molecule.

In a typical reaction, 10 mg of amylose, 2 μmoles of uniformly labeled [^{14}C]-glucose, 5 μmoles phosphate buffer, pH 7.0, and amylomaltase (approx. 100 units) in a total volume of 0.5 ml were incubated for 4 h at 28°. Following this incubation the entire reaction mixture was chromatographed for 20 h on Whatman 3MM filter paper in the ethanol–butanol–water solvent system of PUTMAN AND HASSID[6]. The areas of the chromatogram corresponding to glucose, maltose, maltotriose and other higher oligosaccharides were located by radio-autography, excised, and eluted with water. A typical radioautogram is shown in Fig. 1.

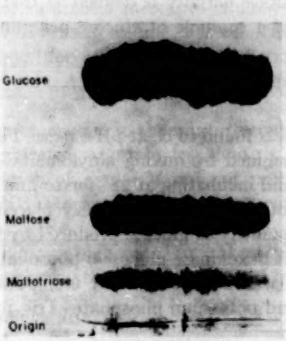

Fig. 1. A radioautogram prepared from a chromatogram of the reaction mixture containing [^{12}C]amylose, [^{14}C]glucose and amylomaltase. The chromatogram was prepared using the ethanol–butanol–water solvent system described in the text.

Preparation of [^{14}C]maltobionic acid: [^{14}C]maltobionic acid was prepared from [^{14}C]maltose by the bromine oxidation procedure of HUDSON AND ISBELL[7] as modified by FITTING AND PUTMAN[8]. The maltobionic acid was isolated by paper chromatography in the propanol–ammonia–water solvent system of HANES AND ISHERWOOD[9]. The bands corresponding to maltose and maltobionic acid were located by spraying marker strips with neutral brom cresol green in absolute methanol. The [^{14}C]bands were identified by radioautography of the chromatograms.

Spray reagents for the location of nonradioactive sugars

The spray reagent described by GARDNER[10] was used to locate the position of the free sugars after chromatography. However, this reagent was found to be inadequate for the detection of the corresponding monomethylated derivatives and a modification of this spray was employed. By replacing the brom thymol blue indicator used in the above spray by cresol red, the presence of the methylated sugars was made quite apparent. The sugar–borate–dye complexes appear yellow on a red background. The cresol red sugar complexes possess the characteristic fluorescence like those of brom thymol blue described by GARDNER[10], thus permitting one to locate the various sugars long after the visible color contrast has faded.

Enzyme preparation

Throughout this study amylomaltase purified as described previously[1] was used. The particular preparation used in the present work had a specific activity of 7000 units/mg protein which is somewhat less than the previously described one[1].

Maltose phosphorylase was prepared according to the procedure of FITTING AND DOUDOROFF[11] from *Neisseria meningitidis* grown in Difco nutrient broth supplemented with 2 % maltose. The enzyme was shown to be free of amylase and maltase activity, and to depend upon the presence of inorganic phosphate for the cleavage of maltose.

*Assay procedure**

One unit of amylomaltase activity is defined as that amount of enzyme which will catalyze the release of 1 mμmole of glucose per min from maltose at 28° and pH 6.9.

EXPERIMENTAL

pH optimum

The optimal activity was found to be at pH 6.9 (see Fig. 2). The enzymic activity at various pH's was determined by mixing amylomaltase, maltose (10 μmoles/ml), and buffer (5 μmoles/ml) and incubating at 28° for 10 min. The reaction was stopped by pipetting 0.5-ml aliquots into tubes previously placed in a boiling water bath. After 3 min heating, the quantity of glucose produced by the amylomaltase reaction was assayed by means of the hexokinase-glucose-6-phosphate dehydrogenase reaction[1]. Potassium acetate buffer was used below pH 6.0, potassium phosphate–acetate between pH 6.0 and 7.0 and potassium phosphate–Tris above pH 7.0.

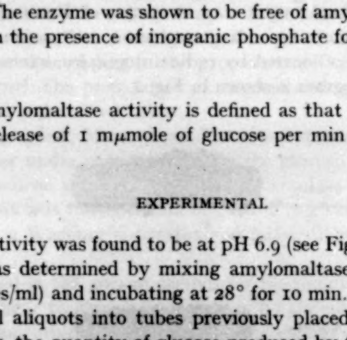

Fig. 2. The effect of pH on amylomaltase activity.

The turnover number of amylomaltase

The turnover number of amylomaltase calculated for a protein of molecular weight 124,000 having a specific activity of 9,400 units/mg protein[1] was found to be 1,160 moles of maltose cleaved/mole of enzyme/min at 28°, pH 6.9.

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* See ref. 1.

Effect of heavy metals on amylomaltase

Heavy metal ions, *e.g.*, $Hg(NO_3)_2$, $NiCl_2$, $CuSO_4$ and $ZnSO_4$, at concentrations of $1 \cdot 10^{-3}\,M$ completely inhibited this enzyme. Ca^{++} and Mn^{++} ions at the above concentration had no effect on its activity. The procedure used for determining the effectiveness of a particular metal ion was to incubate the ion (1 μmole/ml) with enzyme, maltose (2 μmoles/ml) and potassium phosphate buffer pH 7.0 (5 μmoles/ml) in a final volume of 1 ml. After 30 min at 32°, the reaction was stopped by heating to 100° for 3 min and 0.1-ml aliquots were assayed for glucose with appropriate controls to show that the assay system was active.

In addition to the effects of the above metals, *p*-chloromercuribenzoate (*p*CMB) was also investigated for its ability to inhibit amylomaltase. The enzyme was incubated with $2 \cdot 10^{-4}\,M$ *p*CMB in the presence and absence of maltose for various lengths of time. Aliquots of these reaction mixtures were removed and pipetted into tubes containing 2 μmoles maltose, 4 μmoles mercapto-ethanol and 5 μmoles potassium phosphate buffer at pH 7.0 in a final volume of 1 ml. This latter reaction mixture was allowed to incubate at 37° for 10 min, after which time the tubes were placed in a boiling water bath for 3 min. Aliquots of the boiled reaction mixtures were then assayed for glucose.

1 min after the addition of enzyme to the mixture containing *p*CMB, the amylomaltase activity was 84 % inhibited. At 2 min, less than 1 % of the activity remained.

Maltose at a concentration of $4 \cdot 10^{-3}\,M$ did not protect the enzyme from inactivation by $2 \cdot 10^{-4}\,M$ *p*CMB. The enzyme is saturated with respect to its substrate at this concentration of maltose.

Versene at concentrations as high as $3 \cdot 10^{-3}\,M$ at pH 7.0 was ineffective in inhibiting amylomaltase, suggesting that a divalent metal is not required for activity.

The stoichiometry between maltose disappearance and glucose appearance

A reaction mixture was prepared consisting of 0.05 μmoles maltose, 2 μmoles $MgCl_2$, 5 μmoles glycylglycine pH 7.0, 0.4 μmoles TPN, 1 μmole ATP, hexokinase-glucose-6-phosphate dehydrogenase, and amylomaltase in a final volume of 1 ml. After the complete disappearance of maltose, the yield of glucose in two experiments was 0.053 and 0.049 μmoles.

These data confirm the earlier work of MONOD AND TORRIANI[12] and further indicate that the purified enzyme is free of both maltase and amylase activities. The presence of the former enzyme would have resulted in the release of two glucose units per maltose molecule cleaved, while the latter would have led to a value of greater than one and less than or equal to two.

Equilibrium constant of amylomaltase

Starting with maltose as substrate, a reaction mixture containing 5 μmoles maltose, amylomaltase and 5 μmoles glycylglycine buffer pH 6.9 in a total volume of 2 ml was incubated at 28° and 0.1-ml aliquots were removed at 0, 30, 60, 120 and 240 min. The aliquots were pipetted into tubes previously placed in a boiling water bath. Glucose was determined on portions of the heated solution. Controls were run to show that glucose could be quantitatively recovered under the conditions of the experiment. At equilibrium 0.164 μmoles glucose per 0.1-ml aliquot was released. This value was attained at 28° in 30 min.

If one assumes the value of 4 glucose units per polysaccharide molecule formed at equilibrium[3], the balanced equation for the reaction can be written as shown below:

$$\text{n maltose} \rightleftharpoons \text{n glucose} + \text{n}/4\ (\text{glucose})_4$$

The equilibrium constant would then be written as:

$$K = \frac{[\text{glucose}]\ [\text{polysaccharide}]^{1/4}}{[\text{maltose}]}$$

Since the cleavage of maltose results in the release of free glucose and the formation of the polymer, the concentration of the polymer could be assumed equal to [glucose]/4 or 0.041 μmoles. The maltose concentration at equilibrium was determined by difference, *i.e.*, $0.250 - 0.164 = 0.086$.

Under these conditions the equilibrium constant for a unit chain length of 4 was found to be 0.8. If 5 and 6 unit chain lengths are assumed, the equilibrium constants are 0.96 and 1.12, respectively.

The value of 0.52 for the ratio of maltose to glucose at equilibrium is to be compared with the reported value 0.6 given by MONOD *et al.*[12].

Mechanism of polymer formation

Since one glucose molecule is released for each molecule of maltose acted upon by amylomaltase, the question is raised concerning the mechanism of the polymer formation, *i.e.*, which of the two glucose units is released as free glucose and which goes toward the formation of the polymer?

Considering the reaction in the direction of maltose formation, two mechanisms are postulated for the formation of maltose from amylose and glucose. These reactions are schematically shown in Fig. 3.

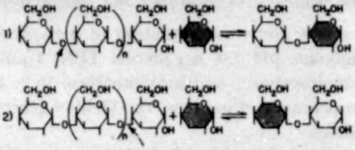

Fig. 3. Two possible mechanisms proposed for the formation of maltose from amylose and [^{14}C]-glucose as catalyzed by amylomaltase. The shaded molecules represent the radioactive components of the reaction mixture described in the text.

In the first scheme, the non-reducing end of the amylose molecule gives rise to the non-reducing end of the maltose formed. The alternative scheme indicated that the reducing end of amylose forms the reducing end of maltose. In both mechanisms the α-1,4 glycosidic bond of amylose is cleaved and the energy of the linkage in the polysaccharide is retained in the maltose.

The principle of the experiment used to decide which of the above reactions is catalyzed by amylomaltase was as follows:

Maltose was isolated from the amylomaltase catalyzed reaction of uniformly labeled [^{14}C]glucose and [^{12}C]amylose. The glucose units constituting the reducing and non-reducing ends of maltose were separated and their specific radioactivity determined. If the non-reducing end of amylose gave rise to the non-reducing end of maltose, then the reducing end of the maltose would be expected to contain all the radioactivity. If, on the other hand, the reducing end of amylose formed the reducing end of maltose, then the non-reducing end of maltose would contain all the radioactivity.

The experimental conditions as well as the technique for isolating the radioactive maltose is described in the methods section. The specific activity of the glucose used for the preparation of the labeled maltose and that of the maltose isolated from the reaction mixture were identical and equal to $3.2 \cdot 10^{-5}$ counts/min/µmole.

This finding implies that only one-half of the maltose molecule was radioactive. In order to determine in which glucose unit the radioactivity was located, the [^{14}C]-maltose was degraded by two methods. These were the specific enzymic cleavage of maltose by maltose phosphorylase[8], and the chemical oxidation of maltose to maltobionic acid and its subsequent hydrolysis to glucose and gluconic acid[7].

Cleavage of [^{14}C]maltose by maltose phosphorylase: The reaction catalyzed by maltose phosphorylase was demonstrated by FITTING AND PUTMAN[8] to proceed as shown in Fig. 4, line 1. As indicated, radioactive β-glucose-1-phosphate would result from cleavage of maltose labeled in the non-reducing end of the molecule (line 2), while [^{14}C]glucose would be released if the molecule were labeled in the reducing end (line 3).

The following experiment showed that the radioactive glucose constituted the reducing end of the isolated maltose. A reaction mixture containing 0.16 µmole of [^{14}C]maltose, 5 µmoles of potassium phosphate buffer pH 7.0, and the enzyme, maltose phosphorylase, in a total volume of 1.0 ml was incubated at 37°. At 0, 60 min, 210 min and 270 min, 0.1-ml aliquots of the reaction mixture were placed on Whatman 3MM filter paper and chromatographed in the butanol–ethanol–water solvent system for 20 h. At 280 min the remaining 0.6 ml was chromatographed as described above. Fig. 5 is a photo of the radioautogram and illustrates the release of [^{14}C]glucose with time. The presence of non-radioactive β-glucose-1-phosphate was

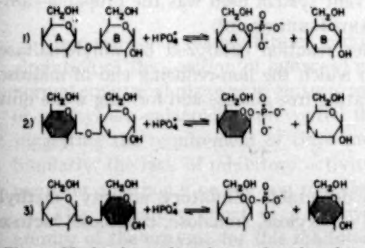

Fig. 4. Mechanism of maltose phosphorylase action[11]. Line 1 represents the mechanism of maltose phosphorylase cleavage. Lines 2 and 3 are the results expected from the cleavage of maltose labeled in either the non-reducing (line 2) or the reducing (line 3) end of the molecule. The shaded molecules represent the radioactive components of the reaction mixture described in the text.

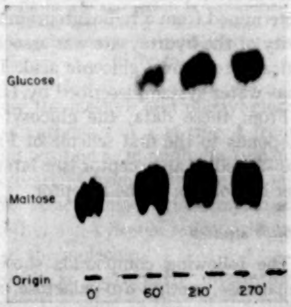

Fig. 5. Radioautogram illustrating the cleavage of specifically labeled maltose by maltose phosphorylase. Aliquots of a reaction mixture containing specifically labeled [^{14}C]maltose, inorganic phosphate and maltose phosphorylase were removed at the times indicated, spotted on Whatman 3MM filter paper and chromatographed for 20 h in the ethanol–butanol–water solvent system described in the text.

shown by spraying with the phosphate reagent of WADE AND MORGAN[13]. The phosphate ester appeared as a spot near the origin of the chromatogram and was found to possess less than 5 % of the total radioactivity.

The glucose and maltose of the 280 min reaction mixture were located by radioautography of the chromatogram. After removing these areas, the sugars were eluted with distilled water. The concentration of free glucose was determined by means of the hexokinase glucose-6-phosphate dehydrogenase assay system. The maltose concentration was determined by adding amylomaltase to the above assay system.

The specific radioactivity of the glucose and maltose isolated from the chromatogram was $2.6 \cdot 10^5$ and $3.1 \cdot 10^5$ counts/min/μmole. The initially chromatographed maltose had a specific activity of $3.2 \cdot 10^5$ counts/min/μmole.

The free glucose produced by the maltose phosphorylase reaction had 82 % of the specific radioactivity expected if only the reducing end of the maltose molecule were labeled. The total glucose and maltose recovered was 80 % of the starting material.

Since the nonreducing end of the maltose molecule was not labeled, the value of 82 %, although lower than expected, is sufficiently high to permit the conclusion that the original maltose possessed its radioactivity in the reducing end of the molecule.

Hydrolysis of [^{14}C]maltobionic acid: Maltobionic acid was prepared from the radioactive maltose described above by the bromine oxidation procedure described by HUDSON AND ISBELL[7]. The results of chemical degradation of this maltobionic acid offered qualitative confirmation of the findings with enzymic cleavage.

Assuming the label to be present in the reducing end of the maltose molecule, one would expect the gluconic acid arising from the acid hydrolysis of maltobionic acid to be radioactive while the glucose would be nonradioactive.

Chromatography of the maltobionic acid hydrolysate showed the glucose to be nonradioactive (*i.e.*, less than 1 % of that represented by the gluconic acid spot), as determined from a radioautogram of the chromatographed hydrolysate. The radioactivity of the hydrolysate was associated with a compound which chromatographed identically to known gluconic acid. The solvent system used was the propanol–ammonia–water system described by HANES AND ISHERWOOD[9].

From these data, the glucosyl transfer reaction catalyzed by amylomaltase corresponds to the first scheme of Fig. 3 in which the non-reducing end of maltose is transferred to an acceptor (see later) liberating free glucose and forming a one unit higher homologue of the acceptor.

Inhibitor studies

The following compounds showed no detectable inhibitory activity: methyl α-D-mannose, methyl α-D-galactose, methyl α-D-xylose, trehalose, cellobiose, lactose and sucrose.

The following compounds acted as competitive inhibitors: methyl α- and β-D-glucose, phenyl α- and β-D-glucose and β-methyl maltoside.

Table I gives the K_i for each of the competitive inhibitors and Fig. 6 illustrates the detailed analysis of the most active of them, β-methyl maltoside. The K_i for β-methyl maltoside was found to be $2.5 \cdot 10^{-3}$ M in glycylglycine buffer ($5 \cdot 10^{-2}$ M) pH 7.0 at 28°. The K_s for maltose calculated from the LINEWEAVER-BURK[14] plots of

$1/v$ *vs.* $1/s$ was found to be $1.7 \pm 0.2 \cdot 10^{-3}$ *M*, an average of four determinations.

The structure shown in Fig. 7 is a diagrammatic representation of the maltose molecule and is included to aid in the discussion of the inhibitors.

TABLE I

COMPETITIVE INHIBITORS OF AMYLOMALTASE

Compound	K_i
β-methyl maltoside	$2.5 \cdot 10^{-3}$ *M*
Methyl α-D-glucoside	$8 \cdot 10^{-3}$ *M*
Methyl β-D-glucoside	$1 \cdot 10^{-2}$ *M*
Phenyl α-D-glucoside	$1 \cdot 10^{-2}$ *M*
Phenyl β-D-glucoside	$3 \cdot 10^{-2}$ *M*

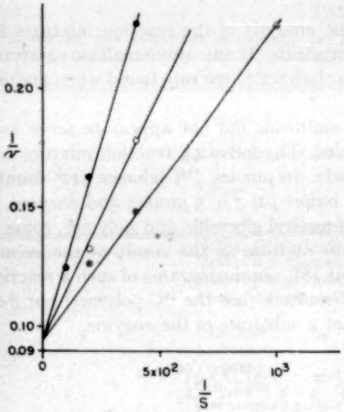

Fig. 6. Lineweaver-Burk plot illustrating the competitive inhibition of amylomaltase by β-methyl maltoside with maltose as substrate: *s* = substrate concentration (moles/l); *v* = enzyme activity (units). Concentration of β-methyl maltoside: ●—● $5 \cdot 10^{-3}$ *M*; ○—○ $1 \cdot 10^{-3}$ *M*; ⊙—⊙ none added.

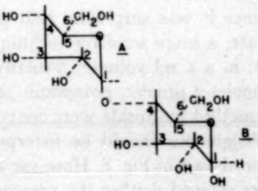

Fig. 7. A representation of the maltose molecule. The dotted lines illustrate the bonds to groups which lie in a plane which is equatorial to the plane of the respective glucose rings. The solid lines lead to groups perpendicular to the plane of the ring.

In contrast to the inhibitions manifested by the methyl- and phenyl-glucosides, alteration of the position of hydroxyl groups of carbons 2 or 4 of ring A from their normal equatorial plane as in glucose, to an axial position as in methyl α-D-mannoside or methyl α-D-galactoside, prevented these derivatives from inhibiting the enzyme, suggesting the requirement of these groups for attachment to the enzyme surface. Similarly, the lack of inhibitory activity of methyl α-D-xyloside reflects the requirement for a carbon 6 on the non-reducing end of the molecule.

The inhibitory effect of β-methyl maltoside is particularly interesting. The affinity of the enzyme for this inhibitor is about the same as that of the substrate, maltose. The inhibition of amylomaltase by this maltose analogue would suggest that the methyl substituted carbon 1 of ring B does not interfere with the attachment of this molecule to the active site of the enzyme.

Alteration of the glycosidic linkage from the α-1,4 found in maltose to β-1,4 as in cellobiose results in this substance showing no inhibitory effect on amylomaltase. Similar alterations of the glycosidic linkage from α-1,4 to α, α′-1,1 as in trehalose,

α-1,2 as in sucrose, α-1,6 as in dextran, or β-1,4 as in lactose resulted in these substances lacking inhibitory activity.

The conclusions drawn from the above study regarding attachment of the substrate to the active site of amylomaltase are: first, the non-reducing end of the disaccharide must resemble glucose in its arrangement of hydroxyl groups (except at carbon 3, for which no evidence for or against its effect is known); second, the glycosidic linkage between glucose and a carbohydrate aglycone must be α-1,4; and third, the hydroxyl group on the reducing end of the disaccharide is not required for attachment to the active site.

Substrate studies and their relevance to the active site of amylomaltase

None of the compounds (listed previously) tested for inhibitory activity acted as substrates in the reaction

$$\text{n glucose-x} \rightleftharpoons \text{nx} + (\text{glucose})_n$$

This conclusion is based on chromatographic analysis of the reaction mixtures for polymer or altered forms of the presumed substrate. If any amylomaltase catalyzed reaction did occur, it would have to be at less than 0.1 % the rate found when maltose is a substrate.

Since it was surprising that β-methyl maltoside did not appear to serve as a substrate, a more sensitive technique was used. The following reaction mixture was set up: in a 1 ml volume: β-methyl maltoside, 10 μmoles, [^{14}C]glucose (10^6 counts/min/μmole) 2 μmoles, potassium phosphate buffer pH 7.0, 5 μmoles and enzyme. If any β-methyl maltoside were converted to β-methyl glucoside and polymer, some of the [^{14}C]glucose should be incorporated into maltose as the result of the second reaction shown in Fig. 8. However after 4 h at 28°, chromatograms of such a reaction mixture showed neither the presence of [^{14}C]maltose (nor the ^{14}C polymer) nor β-D-glucoside. Therefore β-methyl maltoside is not a substrate of the enzyme.

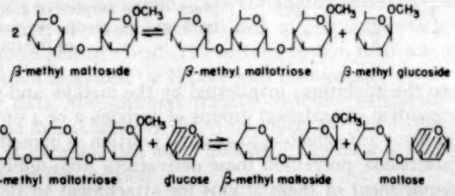

Fig. 8. Illustration of a mechanism by which [^{14}C]maltose might be expected to arise in the reaction between β-[^{12}C]methyl maltoside and [^{14}C]glucose (shaded molecule). The experiment testing this hypothesis is described in the text.

It should be noted that in the above studies one is measuring the ability of a substance to serve as a substrate in the forward reaction, *i.e.*, cleavage of n glucose-x to give $(\text{glucose})_n + \text{nx}$. Although it was not possible to show substrate activity with any of the compounds tested, it was of interest to determine whether any of these could serve as glucosyl acceptors in the reverse reaction. In this way, one could obtain information regarding the specificity, if any, of the reducing end of the amylomaltase substrate.

A typical reaction mixture for such studies contained 10 mg of amylose, prepared enzymically, 20–100 μmoles glucosyl acceptor, 5 μmoles potassium phosphate buffer pH 7.0, and purified enzyme in a total volume of 1.0 ml. The reaction mixtures were allowed to incubate for 4 to 8 h at 28° and then chromatographed on Whatman 3MM filter paper using the ethanol–butanol–water solvent system described in the METHODS section. Of the compounds tested, only glucose, β-methyl maltoside and mannose were able to serve as glucosyl acceptors (see Table II). The appearance on the chromatogram of compounds having mobilities different from that of either amylose or the glucosyl acceptors was presumed to be the β-methyl maltotriose and glucomannoside, respectively. Under the conditions of the experiments, the amylose was not degraded unless a glucosyl acceptor was added to the reaction mixture, *i.e.*, in the absence of acceptor only the added carbohydrates appeared after development of the chromatogram.

TABLE II

A SURVEY FOR GLUCOSYL ACCEPTORS (X) IN THE REACTION AMYLOSE + X $\rightleftharpoons$ GLUCOSE-X

Glucosyl acceptor (x)	*Formation of glucose-x*
Glucose	+
Mannose	+
β-methyl maltoside	+
Xylose	—
Methyl α-D-mannoside	—
Methyl β-D-xyloside	—
Methyl α-D-glucoside	—
Methyl β-D-glucoside	—
Phenyl α-D-glucoside	—
Galactose	—
Lactose	—
Trehalose	—
Cellobiose	—
Melibiose	—
Melizitose	—
Arabinose	—
Ribose	—
Dextran + Glucose	—

+ = Detectable reaction as described in text.
— = No detectable reaction.

Previous findings of DOUDOROFF *et al.*[3] also suggested the role of mannose as a glucosyl acceptor for the amylomaltase of *E. coli*. The compounds tested in addition to β-methyl maltoside and mannose are listed in Table II.

From the data obtained as a result of these studies, the following restrictions might be placed on the reducing end of the disaccharide serving as substrate for amylomaltase:

1. The hexose unit accepting the glucosyl moiety must possess either a free hydroxyl group on the reducing carbon, or if it exists as a part of a disaccharide, the glycosidic linkage must be α-1,4.

2. The hydroxyl group at position 2 can exist as either the equatorial or axial form and is presumed not to be concerned with attachment of the acceptor to the active site of the enzyme.

3. The hydroxyl group at carbon 4 must be equatorial, since galactose is not a glucosyl acceptor.

4. The carbon at position 6 is necessary for the hexose serving as acceptor, since xylose does not meet these requirements. This result has yet to be clarified since DOUDOROFF *et al.*[3] have found xylose to be an acceptor, albeit a poor one.

When dextran, the α-1,6 polysaccharide, was examined for its ability to serve as glucose donor, it was found to be inert (*i.e.*, it was not possible to show the formation of maltose using dextran, as glucose donor, and glucose as the glucosyl acceptor).

To summarize the findings of the above studies, the disaccharide, by the specificity of amylomaltase, is shown in Fig. 9.

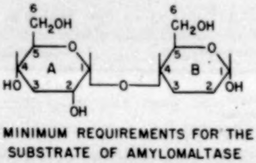

Fig. 9. The substrate and inhibitor studies suggested the requirement for equatorial hydroxyl groups at carbons 2 and 4 of ring A, an unsubstituted carbon at position B-1 and the required presence of the 6 carbon of both rings A and B.

If one designates the non-reducing end of the molecule as A and the reducing end as B, then a substrate for the enzyme must have the hydroxyl groups of ring A at carbons 2 and 4 in the equatorial plane of the ring. Carbon atom 6 in ring A must be present. The glycosidic bond must be α-1,4; the hydroxyl group must be present at carbon 1 in ring B; the terminal carbon 6 in ring B must also be present.

DISCUSSION

The transfer of a glycosyl unit to an acceptor is known to be catalyzed by three categories of enzymes: the hydrolases, which transfer primarily to water, the phosphorylases which use inorganic phosphate or arsenate as acceptor, and the transglycosidases which utilize, generally, carbohydrates as acceptor. Whereas the hydrolyases and some phosphorylases will under certain conditions transfer to carbohydrate, the strict transglycosidases, of which amylomaltase is an example, will use only a carbohydrate as acceptor.

It has been shown that the reaction catalyzed by amylomaltase involves the transfer of the glucosyl moiety forming the non-reducing end of the substrate, to an acceptor. In this respect the mechanism of action of amylomaltase resembles that of the D-enzyme described by WALKER AND WHELAN[15]. Both these enzymes transfer the non-reducing end of the substrate to an acceptor, but differ in that the D-enzyme, a disproportionating transglucosidase, transfers not one, but several hexose units at a time.

The polymer formed by the action of amylomaltase on maltose (*i.e.*, amylose) retains the α-1,4 glycosidic bond present in maltose. Furthermore, maltose is the only disaccharide formed by the amylomaltase catalyzed reaction between glucose and amylose. If the transfer of the glucosyl unit by amylomaltase were to occur in a single step, the product would not be expected to retain the configuration of the glycosidic linkage of the substrate. The single displacement reaction has been postulated to explain the reaction catalyzed by maltose phosphorylase in which the α-linkage of maltose become the β-linkage of the product, β-glucose-1-phosphate. Since the configuration is retained in the case of amylomaltase, one would predict the transfer to occur in two stages. KOSHLAND[16] has discussed two mechanisms whereby an enzyme could catalyze a transfer without change in anomeric configuration and these need not be elaborated here.

From the substrate studies on amylomaltase, one can conclude that the enzyme has at least one stereospecific site for the glucosyl residue which it transfers. The question could be asked, "Does the enzyme have any specificity with regard to acceptor, *i.e.*, is it necessary to postulate a second stereospecific site?".

It was pointed out in the earlier part of the discussion that amylomaltase does not transfer the glucosyl unit to water or inorganic phosphate. While this observation in itself implies another specific site which recognizes the acceptor, the argument is strengthened by the fact that galactose, fructose, and cellobiose, which are closely related to the acceptors, will not react in this system. However, the description of the specificity of the enzyme is clearly insufficient to establish this "two site hypothesis", and other kinetic and chemical experiments will have to be done.

ACKNOWLEDGEMENTS

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THE CHARACTERIZATION OF THE PATHWAY OF MALTOSE UTILIZATION BY *ESCHERICHIA COLI*

III. A DESCRIPTION OF THE CONCENTRATING MECHANISM

HERBERT WIESMEYER[*], [**] AND MELVIN COHN[***]

Department of Microbiology, Washington University School of Medicine, St. Louis, Mo. (U.S.A.)

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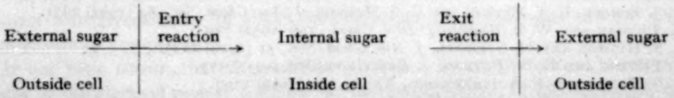
SUMMARY

Maltose has been shown to be concentrated in *E. coli* via an inducible system, termed permease, analogous to that described for galactosides[3]. The activity of the permease is temperature dependent, there being an 80-fold difference in rates between 0° and 37°. Furthermore, the uptake is blocked by sodium azide, indicating the dependence of activity on an energy generating system. The internally accumulated carbohydrate is largely maltose but other compounds are detectable. Although the internal carbohydrate cannot be removed from the cell by washing, it exchanges readily with externally added maltose. Mutants of *E. coli* which are selected because they are resistant to bacteriophage λ are also found to be maltose-negative because they lack the permease. This finding suggests a relationship between virus receptor sites and the maltose entry system.

INTRODUCTION

The first two papers[1, 2] in this series have described the enzyme amylomaltase which catalyzes the conversion of maltose to glucose and amylose. However, this is not the first reaction in the utilization of maltose by *Escherichia coli*. Maltose is concentrated in the cell by an active transport mechanism which will be termed the maltose permease. The properties of this concentrating system will be described here and a comparison will be made between the maltose permease and those previously described for other carbohydrates[3].

It might be recalled that the process by which certain sugars are accumulated in the cell might be represented as follows[3]:



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[*] This work is a part of the thesis submitted by H. WIESMEYER to the Graduate School of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[**] Present address, School of Biology, Johns Hopkins University, Baltimore, Maryland.

[***] Present address, Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California.

The externally added carbohydrate is concentrated in the cell *via* the entry permeability factor called permease and leaves the cell *via* an independent reaction to become external sugar again. The steady state concentration of internal sugar is dependent upon the relative rates of the entry and exit reactions. However, the initial rate of accumulation of internal carbohydrate measures the entry reaction.

The analysis of a concentrating mechanism in a bacterial cell population requires that the test compound, which is concentrated within the cell, not be further metabolized. Two techniques for accomplishing this are evident: (a) the use of a compound whose structure is such that it is concentrated by the system to be studied, but is not a substrate of any enzyme in the cell; or (b) the use of a bacterial strain which possesses the permease but is unable to synthesize the enzyme (or enzymes) which act on the concentrated compound. Since there are no known compounds which are concentrated by the permease but are not further metabolized, the second technique was employed. A mutant was used which lacked the ability to synthesize amylomaltase but possessed an inducible maltose concentrating system. The essential assumption implied by this choice is that amylomaltase is the only enzyme present in *E. coli* which catalyzes a reaction involving maltose. While our data indicate that this assumption may not be entirely valid, the fact that about 60 % of the maltose concentrated within the bacteria can be recovered as free maltose indicates that this mutant system is a practical if not ideal means of studying the maltose permease.

METHODS AND MATERIALS

Bacterial strain

The maltose negative strain used in these studies, *E. coli* W102, was obtained from Dr. J. MONOD. This strain was originally derived from the Y-10 strain of *E. coli*, known to be lysogenic for phage λ and to require threonine, leucine and vitamin B_1 for growth (personal communication from Dr. J. LEDERBERG). This strain possesses an inducible permease and less than 0.1 % of the amylomaltase found in the wild type maltose positive strain.

Growth medium

E. coli W102 was routinely grown in synthetic Medium 56[3] supplemented with threonine (5 μg/ml), leucine (5 μg/ml) and vitamin B_1 (0.06 μg/ml) using succinate as the sole carbon and energy source. When referred to in the text, this medium will be designated as M56TLB$_1$S. Maltose (Mal), at a final concentration of $5 \cdot 10^{-4}$ *M* is added to the above medium for the induction of the permease.

In preparing M56TLB$_1$SMal, the supplements were autoclaved separately and individually added to the sterile synthetic basal medium.

Preparation of [^{14}C]maltose

Two sources of radioactive maltose were used: (a) commercial "research quality" uniformly-labeled maltose from Nuclear-Chicago and (b) maltose synthesized from the amylomaltase-catalyzed reaction between uniformly labeled glucose and unlabeled amylose[2].

The commercial maltose was contaminated with radioactive glucose, maltotriose and higher homologues. These impurities were separated from the maltose by chromatography[4].

Assay for accumulated "maltose"

The method employed for the determination of the internal "maltose" was based on the uptake of [^{14}C]maltose by the induced culture. The procedure followed is described below: Induced cells grown in M56TLB$_1$S + 10^{-2} *M* maltose were centrifuged, washed once with M56 and resuspended in fresh M56 with 10^{-3} *M* succinate, [^{14}C]maltose ($1 \cdot 10^{-3}$ *M*) and chloramphenicol (40 μg/ml). The cells were then placed in a flask in a rotary shaking water bath at 37° and aliquots of the culture removed at various times. These aliquots were passed through an HA millipore filter (Millipore Filter Corporation, Watertown, Mass.) under suction, washed with 10 ml of cold distilled water and air dried on the filter set-up. The dried filters were then glued to copper plancets and the radioactivity determined using a windowless gas flow counter (Nuclear-Chicago Model 127). One unit of activity of the concentrating system will be defined as 1 mμmole "maltose" accumulated/min under these standard conditions where the initial rate is measured.

As will be seen later, the internal radioactivity measured here consists of maltose itself and some other substances derived from maltose. For this reason the internal carbohydrate is referred to as "maltose".

EXPERIMENTAL

Kinetics of [^{14}C]maltose uptake by pre-induced W102

The uptake of [^{14}C]maltose by cells previously grown in the presence of maltose is shown in Fig. 1. A plateau in the uptake of [^{14}C]maltose was found to occur 90 to 120 min after the cells had been placed in the assay medium. At this point, the internal concentration of radioactivity corresponded to approximately 600 mμmoles of "maltose"/mg wet wt. of cells. In other words, the internal concentration of sugar is 29 % by weight assuming the cell weight to be 75 % of the measured wet wt.

Effect of temperature on the uptake of [^{14}C]maltose

The uptake of [^{14}C]maltose as a function of temperature was investigated using pre-induced cells suspended in the assay medium and incubated at 0° and 37°.

Maltose uptake was found to be temperature dependent (see Fig. 2). The culture maintained at 37° attained a maximum internal "maltose" concentration (as determined by the uptake of radioactivity) 90 min after coming into contact with the

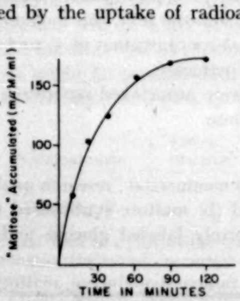

Fig. 1. Kinetics of uptake of [^{14}C]maltose by *E. coli* W102.

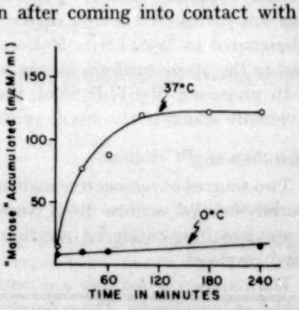

Fig. 2. Temperature effect on the uptake of [^{14}C]maltose.

radioactive sugar, whereas the 0° culture had taken up less than 10 % of this amount in that time. There is an 80-fold difference in initial rate corresponding roughly to a Q_{10} of 3.

The cells remaining at 0° for 90 min were shown to be fully capable of accumulating "maltose". 15 min after the bath temperature had been increased to 37°, these cells had taken up an equivalent of 180 mμmoles of maltose/mg wet wt. of cells. This value corresponds to the initial rate of maltose uptake by the cells maintained at 37°.

The dependence of maltose uptake on the availability of an energy generating system

An attempt to demonstrate an energy dependence for the uptake of maltose via the concentrating system was undertaken in the presence of 10^{-2} *M* sodium azide. Aliquots for the assay of accumulated "maltose" were removed at 30, 60, 120, 180 and 240 min after the addition of a fully induced culture to the assay medium. The uptake of maltose by the azide treated culture as compared to its uptake by a non azide treated culture is shown in Fig. 3.

The initial rate of maltose uptake in the presence of sodium azide was 30 % of that found in its absence. The kinetics of this uptake were not changed by preincubation of the cells with azide for 15 min prior to the addition of [^{14}C]maltose.

Properties of the internal "maltose"

In contrast with previously studied systems[3], the internal sugar could not be removed by repeated washing either on the millipore filter or by resuspension and centrifugation.

Extraction of the major portion of the internal radioactivity can be accomplished with hot water (repeated 10 min incubations at 100°). By means of this procedure, 83 % of the residual radioactivity was removed. Of the remaining 17 %, resistant to extraction with hot water, all but 2 % was soluble in hot 4 % perchloric acid.

Warm 75 % ethanol (45–55°) served as effectively as boiling water in the extraction of the major radioactive fraction. However, here, as in the water extraction procedure, a residual 10 to 20 % of the radioactivity remained with the cells.

Chromatography of the hot water extractable radioactivity, in the ethanol–butanol–water solvent system[4] revealed the presence of two radioactive components. One of the two components, maltose itself, was identified not only by its migration in the solvent system used, but also by its ability to serve as a substrate for amylomaltase. The second component which remained at the origin of the chromatogram has not yet been identified. The first component comprised 60 % of the total hot water extractable material.

The culture supernatant of pre-induced *E. coli* W102 which has actively concentrated "maltose" does not have detectable radioactivity associated with anything but maltose.

Displacement of internal radioactivity by externally added [^{12}C]maltose and [^{12}C]glucose

Although the internal "maltose" could not be washed from the cell, part of it could be displaced by externally added sugars. The specificity of this displacement reaction was studied by using a fully induced culture prepared by growth overnight in maltose. The cells were placed in the usual assay mixture containing [^{14}C]maltose (10^{-3} *M*) and the uptake of radioactive maltose was followed (see Fig. 4).

After 180 min at 37°, these cells had accumulated approximately 600 mμmoles of radioactive "maltose" per mg wet wt. of cells. At this point, the culture was divided into four 1-ml aliquots and non-radioactive glucose, maltose, mannose and β-methyl maltoside was added to the separate tubes at a final concentration of 10^{-1} *M*, *i.e.*, 100 times the external [^{14}C]maltose concentration. At 30 and 60 min after the addition of unlabeled carbohydrate, aliquots were assayed for internal residual radioactivity.

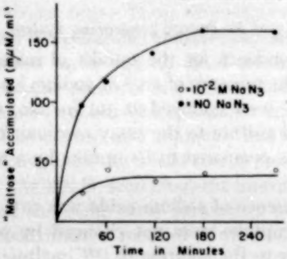

Fig. 3. Effect of sodium azide on [^{14}C]maltose uptake.

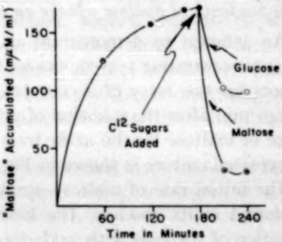

Fig. 4. Displacement of [^{14}C]maltose by [^{12}C]-maltose and [^{12}C]glucose.

The results for glucose and maltose are shown in Fig. 4.

The level of the internal radioactivity, considered as 100 %, was reduced by non-radioactive glucose and maltose to 55 and 15 %, respectively. The other sugars had a much lesser effect leaving the level at 80 % or greater.

In the case of maltose only 1 % of the accumulated sugar should have remained, if the internal and external forms were fully exchangeable. The non-exchangeable fraction, which is 15 % of the total accumulated "maltose", is of the same order of magnitude as the fraction not extractable by hot water. These two components might, therefore, be identical. The material which leaves the cell is chromatographically maltose.

If the extent of the maltose displacement reaction is corrected for the non-exchangeable fraction so that the reaction goes to equilibrium (99 % displacement), then glucose, by comparison, displaces 60 %. The behavior of glucose could be understood, if it were to inhibit the uptake reaction with a subsequent fall in level of total "maltose" accumulated. This is the finding discussed next.

Effect of other carbohydrates on the uptake of maltose

The inhibitory effect of glucose, galactose and β-methyl maltoside on the activity of the maltose-concentrating system was investigated using pre-induced W102. Both [^{14}C]maltose and [^{12}C]sugars were added simultaneously to 1-ml aliquots of culture suspended in M56S containing 40 μg of chloramphenicol. These cultures were incubated aerobically at 37°. 15 min after the addition of the carbohydrates aliquots were removed, passed through the millipore filters, washed, dried and counted. The results are given in Table I, where it can be seen that only glucose inhibits the uptake of maltose to a degree which could account for its action in displacing internal "maltose", thus supporting the above hypothesis.

TABLE I

EFFECT OF OTHER CARBOHYDRATES ON PERMEASE ACTIVITY

Carbohydrate (10^{-3} M) addition to pre-induced W102 + 10^{-3} M [^{14}C]maltose	*Permease activity units/mg wet wt. cells*	*Per cent inhibition*
None	20.0	0
Glucose	10.5	48
β-methyl maltoside	17.3	13
Galactose	17.3	13

The induction of the maltose-concentrating system

From the data available it appears as though the initial rate of maltose accumulation would be a measure of the activity of the uptake reaction. Using such an assay then, the kinetics of the induction of permease was measured after the addition of maltose (10^{-3} *M*) to *E. coli* W102 growing exponentially in M56TLB$_1$S. Aliquots were removed and assayed for "permease" activity. The appearance of induced concentrating activity follows a linear differential rate of synthesis[5] (see Fig. 5) similar to that found for the induced galactoside permease.

The relationship between the utilization of maltose by E. coli *K12 and its susceptibility to bacteriophage λ*

E. LEDERBERG[6] had observed that λ-resistant mutants derived from a sensitive stock no longer utilized maltose. Using this observation, a λ-resistant mutant of *E. coli* W102 was isolated by using the virulent mutant of λ C50. This was done by selecting colonies arising in the area of lysis following infection of W102 by λ C50. The *E. coli* W102/λ was shown to be unable to absorb λ-bacteriophage. This mutant W102/λ, λ-resistant in contrast to strain W102, λ-sensitive, did not possess a detectable maltose "permease" as shown in Fig. 6.

It appears therefore that the correlation E. LEDERBERG[6] observed between λ-resistance and maltose utilization involves the maltose permease specifically.

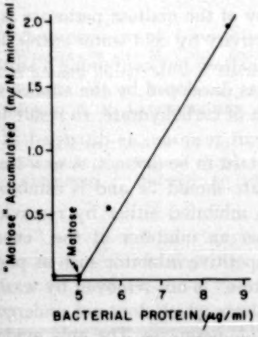

Fig. 5. Induction of the maltose "permease" of *E. coli* W102.

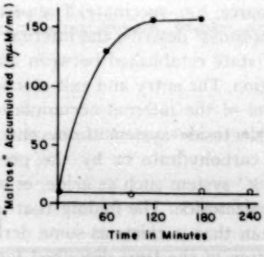

Fig. 6. Uptake of [^{14}C]maltose by λ-resistant and λ-sensitive *E. coli* W102.

DISCUSSION

There are at least 10 independently acting permeases which have been described for the transport of various substances, *e.g.*, carbohydrates, amino acids, etc. into the *E. coli* cell. The maltose-concentrating mechanism appears to be another example of a permease[3]. This system is distinct from the galactoside, galactose and glucose permeases. This conclusion is based not only on the specificity studies but also on the finding that *E. coli* mutants which lack maltose-concentrating activity and therefore cannot grow on maltose will utilize galactosides, galactose, and glucose as sole carbon sources. The converse is also true.

Since the maltose-concentrating system described here differs in some respects from the previously analyzed galactoside-concentrating system[3], the model developed by COHEN AND MONOD should be applied cautiously until it is known whether these differences do actually result from the technical limitations found in working with the maltose permease. The two most important limitations are (a) the lack of an inducer which is a substrate of the permease and yet not metabolizable as a carbon source and (b) the inability to demonstrate a state of crypticity for this enzyme, *i.e.*, to show the presence of amylomaltase in a cell which lacks the maltose-concentrating system.

The second limitation arises because (a) cryptic mutants are not available which synthesize amylomaltase constitutively but do not possess the concentrating system, and (b) it has not been possible to induce amylomaltase in a cryptic mutant which might be expected to have an inducible enzyme.

The only carefully studied carbohydrate system has been the galactoside-permease using methyl β-D-thiogalactoside as substrate[3]. This substance enters the cell not only via the permease but also via some diffusion mechanism[3,6]. It might be distinguished from lactose for which this latter entry mechanism is of minimal importance. As a result *E. coli* cryptic mutants, which cannot be induced by lactose to synthesize β-galactosidase, respond quite well to the alkyl β-D-galactosides[3,6-8]. Maltose might be more analogous to lactose in its behavior than to alkyl-galactosides. The inability of maltose to enter a cell by a non-permease route could explain why an inducible amylomaltase in permease-negative strains is not found.

One peculiar and thus far unexplained property of the maltose permease is the inability to remove the internal form of the radioactivity by cold water extractions, or by resuspension of the cells in medium lacking maltose but containing a suitable energy source, *e.g.*, succinate. The permease model as developed by the studies with the galactosides[3] describe the internal concentration of carbohydrate, to result from a steady state established between an entry and exit reaction, as discussed in the introduction. The entry and exit reactions are presumed to be distinct. A new steady state level of the internal accumulated carbohydrate should be and is established in the galactoside system if the entry reaction is inhibited either by removal of external carbohydrate or by the presence of either an inhibitor of the "energy-generating" system such as azide, or a specific competitive inhibitor such as phenyl β-D-thiogalactoside. The finding that internal "maltose" is not removed by washing could mean that it exists as some derivative of maltose which does not undergo an exit reaction of the type described for the galactoside-permease. The only evidence to the contrary is, that extracts of the cell show, that a large part of the internal "maltose" is maltose itself. Although not washable from the cell, the internal "maltose" does exchange with externally added maltose and what comes out of the cell is maltose itself. These data indicate that a more complex relationship exists between the entry and exit reaction than has been described for the galactoside-permease.

Of the various carbohydrates tested, only glucose has a significant inhibitory effect on the rate of uptake of maltose. β-methyl maltoside, which is an excellent competitive inhibitor of the enzyme, amylomaltase, does not affect the activity of the maltose concentrating mechanism. Similar specificity relationships between the galactoside-permease and β-galactosidase have also been observed and argue that the two systems are distinct. This is further supported by the same finding described for the galactoside system[3], namely that a single cell can concentrate up to a level of $4 \cdot 10^8$ molecules of "maltose" per cell when it has at maximum only 10^4 molecules of amylomaltase.

The maximum rate of the entry reaction for maltose at 37° is approximately 20 mμmoles/min/mg cells, whereas the maximum amylomaltase activity is 70 mμmoles/min/mg cells at the same temperature. Therefore it looks as though the limiting reaction in this activity of the maltose system is the entry reaction, provided that the activity of amylomaltase in a cell is the same as that of the extracted enzyme.

The relationship between the ability to absorb the bacteriophage λ and the presence of a maltose "permease" is of considerable interest because it suggests that the virus receptor site and the "permease" are closely physically associated. If this were true, a rather direct approach to the isolation and chemical characterization of "permease" might be available.

ACKNOWLEDGEMENTS

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STEREOISOMERIC SPECIFICITIES OF 2,3-BUTANEDIOL DEHYDROGENASES

MARY B. TAYLOR AND ELLIOT JUNI

Department of Bacteriology, Emory University, Atlanta, Ga. (U.S.A.)

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SUMMARY

1. Bacteria that produce 2,3-butanediol as an end product of the fermentation of sugars all form D(—)-acetylmethylcarbinol from pyruvate.

2. 2,3-Butanediol dehydrogenases are stereoisomerically specific. Some dehydrogenases oxidize a hydroxyl group in the D(—) configuration [D(—) dehydrogenase] while other dehydrogenases oxidize a hydroxyl group in the L(+) configuration [L(+) dehydrogenase]. Because *meso*-2,3-butanediol contains hydroxyl groups in the D(—) and L(+) configurations, it serves as a substrate for all 2,3-butanediol dehydrogenases. The configuration of the acetylmethylcarbinol formed from the oxidation of *meso*-2,3-butanediol depends upon whether the hydroxyl group in the D(—) or the L(+) configuration is oxidized.

3. *Bacillus polymyxa* contains a D(—)-2,3-butanediol dehydrogenase. The 2,3-butanediol dehydrogenases from *Aerobacter aerogenes* and *Pseudomonas hydrophila* are L(+) dehydrogenases. *Bacillus subtilis* contains both D(—) and L(+) dehydrogenases.

4. The configuration of the carbon atom of 2,3-butanediol that is not oxidized affects the rate of oxidation.

5. Evidence has been presented for the presence of an acetylmethylcarbinol racemase in *Pseudomonas hydrophila*.

6. Bacteria capable of growing with *meso*-2,3-butanediol, sodium acetate or sodium lactate as a source of carbon and energy contain D(—) dehydrogenases. *Aerobacter aerogenes* which has been grown with *racemic* acetylmethylcarbinol as the carbon source contains a D(—) dehydrogenase in contrast to the presence of an L(+) dehydrogenase when *Aerobacter aerogenes* is grown on sugars.

7. The presence of different combinations of D(—) and L(+) dehydrogenases and acetylmethylcarbinol racemase can explain the occurrence of various combinations of the three isomers of 2,3-butanediol as end products of the fermentation of sugars.

INTRODUCTION

All three isomers of 2,3-butanediol [D(—), L(+) and *meso*] are formed as end products of the fermentation of sugars by various microorganisms[1]. Acetylmethylcarbinol

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The following abbreviations are used in this paper: acetylmethylcarbinol, AMC; 2,3-butanediol, 2,3-BD; diphosphopyridine nucleotide, DPN$^+$.

(AMC), the immediate precursor of 2,3-butanediol (2,3-BD) in all microorganisms so far studied, is reversibly reduced by the catalytic action of diphosphopyridine nucleotide (DPN^+)-linked dehydrogenases[2–4]. 2,3-BD dehydrogenases show stereoisomeric specificity[5,6] for a hydroxyl group in either the L(+) or D(—) configuration. STANIER AND FRATKIN[5], studying the oxidation of the three isomers of 2,3-BD by *Aerobacter aerogenes*, *Bacillus polymyxa* and *Pseudomonas hydrophila*, found that these organisms showed stereoisomeric specificity. It has also been shown[6] that *Acetobacter suboxydans* oxidizes *meso*-2,3-BD to AMC but cannot oxidize L(+)-2,3-BD.

The existence of two 2,3-BD dehydrogenases cannot explain the production of some mixtures of the isomers of 2,3-BD during the fermentation of sugars[1]. The present work is a study of the reactions in several bacteria leading to the formation of the three stereoisomers of 2,3-BD. Evidence will be presented to show that the phenomenon can be explained by the presence of various combinations of an AMC racemase and two stereoisomerically specific 2,3-BD dehydrogenases. A preliminary account of this work has already appeared[7].

METHODS AND MATERIALS

Growth of organisms

Bacillus polymyxa strain ATCC 842 was grown on a solid medium containing 0.5 % $CaCO_3$, 0.3 % proteose peptone (Difco), 0.3 % tryptone, 0.3 % yeast extract, 0.1 % soluble starch and 1.5 % agar. The plates were incubated at 30° and the cells harvested after 18–24 h. Cells harvested after more than a 24-h incubation period were inactive. *Bacillus subtilis* Ford strain, obtained from Dr. I. C. GUNSALUS, was grown on a solid medium containing 1.0 % yeast extract, 0.5 % $CaCO_3$, 3.0 % glucose (autoclaved separately) and 1.5 % agar. The plates were incubated at 30° and the cells harvested after 72 h. *Pseudomonas hydrophila* strain ATCC 11439 was grown in a liquid medium containing 0.5 % yeast extract, 0.4 % $CaCO_3$, 0.01 % $MgSO_4$, 1.0 *M* potassium phosphate buffer pH 7.4 (2.6 ml/l) and 1.0 % glucose (autoclaved separately). The culture was incubated at 37° and the cells were harvested after a 24- to 36-h incubation period. *Aerobacter aerogenes* strain ATCC 8724 was grown in the medium described by SILVERMAN AND WERKMAN[8]. The culture was incubated at 37° and the cells were harvested after a 24- to 36-h incubation period.

Isomers of 2,3-butanediol

2,3-BD, obtained from Distillation Products Industries, was purified by distillation and had an optical rotation of 0.0°. The *p*-nitrobenzoate derivative of the distilled product, prepared according to ROBERTSON AND NEISH[9], had a melting point of 195–196° (uncorr.). The reported melting point of the bis-*p*-nitrobenzoate of *meso*-2,3-BD is 197–198°, while the melting point of the corresponding derivative of *racemic* 2,3-BD is 128°, see ref. [9]. D(—)-2,3-BD, $[\alpha]_D^{25}$ —10.6° ($c = 6.7$, in H_2O), was obtained by ether extraction of the growth medium of *B. polymyxa* followed by fractional distillation. The fraction distilling at 65–70° and at 15 mm Hg was collected. NEISH[10] has shown that *B. polymyxa* forms the D(—) isomer of 2,3-BD exclusively and has reported specific rotations as high as — 13.3° for this isomer. D(—)-2,3-BD is strongly hygroscopic, and CLENDENNING[11] has demonstrated that the specific rotation is markedly reduced by varying amounts of water. The specific rotation of a solution of

D(—)-2,3-BD, $[\alpha]_D^{25}$ —10.6° (c = 6.7, in H_2O), agrees well with the data given by CLENDENNING[11] for a solution of the same concentration of pure D(—)-2,3-BD.

The mixture of the L(+) and *meso* isomers of 2,3-BD, $[\alpha]_D^{25}$ + 0.94° (c = 65.3, in H_2O), was extracted from the growth medium of *Ps. hydrophila*. The mixture of the D(—) and *meso* isomers of 2,3-BD, $[\alpha]_D^{25}$ — 5.6° (c = 0.087, in H_2O), was extracted from the growth medium of *B. subtilis*.

Chemical methods

The products of the enzymic reactions were treated as follows: AMC was purified and concentrated by distilling three-quarters of the original volume of its solution which was saturated with sodium chloride. In order to prevent racemization[13] the solution was acidified with one drop of concentrated hydrochloric acid; control experiments have shown that the specific rotation of AMC is not altered by the distillation procedure. (The distillate constituting one-third of the original volume was found to contain 83 % of the total AMC, and all AMC was recovered when two-thirds of the original volume was collected.) 2,3-BD was purified and concentrated as described in the preceding section.

The quantitative determination of AMC and 2,3-BD was carried out according to the methods of WESTERFELD[14] and DESNUELLE AND NAUDET[15], respectively.

Enzyme preparations and assay

Cell-free extracts were prepared from acetone-dried powders[16] of the different bacteria by suspending 1 g of dried cells in 50 ml of water and treating the suspension for 10 min with a Raytheon 10-kc sonic oscillator. The resulting suspension was centrifuged at 25,000 × g for 10 min at 5° and the precipitate discarded. The activities of the 2,3-BD dehydrogenases in cell-free extracts were measured by the initial rate of reduced diphosphopyridine nucleotide (DPNH) formation, following the optical density increase at 340 mμ in a Beckman spectrophotometer (Model DU). With acetone-dried cell preparations, oxygen uptake in the presence of substrate was measured manometrically[8].

Optical rotation

Polarimetric measurements were made with a Rudolph Model No. 70 polarimeter using a sodium arc light source with a 2 dm polarimeter tube (inside diameter 4 mm).

RESULTS

Isomers of 2,3-butanediol produced during fermentation

The specific rotations of the 2,3-BD samples extracted from the growth media after the fermentation of sugars by the bacteria used in this study are shown in Table I, column 1. These results are similar to the findings of others[1]. D(—)-2,3-BD, formed by *B. polymyxa*, is the only unmixed isomer discovered in all fermentations studied. The low specific rotation of 2,3-BD isolated from the other fermentations has been shown to be due to the presence of the *meso* isomer. Small amounts of *racemic* 2,3-BD have been reported to occur in certain fermentations[1].

TABLE I

SUMMARY OF SPECIFIC ROTATIONS

	Specific rotation of 2,3-butanediol		*Specific rotations of acetylmethylcarbinol*	
	1	*2*	*3*	*4*
Organism	$[\alpha]_D^{25}$ *of 2,3-butanediol formed from glucose**	*Approximate composition of 2,3-butanediol formed from glucose***	$[\alpha]_D^{25}$ *of acetylmethylcarbinol formed from pyruvate****	$[\alpha]_D^{25}$ *of acetylmethylcarbinol formed by oxidation of meso-2,3-butanediol* §
B. polymyxa	—10.6° (c = 6.7, in water)	100% D(—)	—96° (c = 0.128, in water)	+73.5 (c = 0.23, in water)
A. aerogenes	+0.8 to 1.8°§§	5–15% L(+) and 85–95% *meso*	—94° (c = 0.07, in water)	—73.0° (c = 0.077, in water)
Ps. hydrophila	+0.94° (c = 65.3, in water)	7% L(+) and 93% *meso*	—34° (c = 0.11, in water)	—70.5° (c = 0.07, in water)
B. subtilis	—5.7° (c = 0.087, in water)	43% D(—) and 57% *meso*	—78° (c = 0.105, in water)	—23.5° (c = 0.16, in water)

* The data in column 1 were obtained by determining the specific rotation of 2,3-BD extracted from the growth medium of each organism as described in METHODS.

** The percentage of each isomer was calculated from the specific rotations in column 1.

*** The data in column 3 were obtained by determining the specific rotation of AMC, formed from pyruvate by acetone-dried cell preparations, after concentration as described in METHODS.

§ The AMC, formed by oxidation of *meso*-2,3-BD by cell-free extracts, was trapped as the oxime, liberated and concentrated as described in METHODS.

§§ These values are taken from the data given in ref. [1].

Optical rotation of acetylmethylcarbinol produced from pyruvate

It must be kept in mind that pyruvate, produced during the fermentation of sugars by the bacteria studied, is largely converted to AMC, which is the direct precursor of 2,3-BD, see ref. [1]. Since all three isomers of 2,3-BD were found in the growth medium after fermentation of sugars, the optical rotation of AMC formed from pyruvate was measured for each bacterium studied. AMC formed from pyruvate by acetone-dried cells of each of these organisms was found to be levo-rotatory. (Table I, column 3).

Stereoisomeric specificities of 2,3-butanediol dehydrogenases

The stereoisomeric specificity of the 2,3-BD dehydrogenase of each organism was studied by oxidizing *meso*-2,3-BD to AMC with acetone-dried cell preparations and determining the specific rotation of the AMC. AMC, formed during the oxidation of *meso*-2,3-BD, was trapped as the oxime according to the procedure of AUBERT AND GAVARD[2]. The oxime was cleaved with nitrous acid, according to the method of BERL AND BUEDING[12], to yield free AMC. The AMC was concentrated as stated in Methods. The method used to trap and subsequently liberate AMC has been shown to result in some racemization[12].

The results given in Table I, column 4, show that the dehydrogenase of *B. polymyxa* oxidized the hydroxyl group of *meso*-2,3-BD which was in the D(—) configuration [D(—) dehydrogenase] to yield L(+)-AMC (Fig. 1). The dehydrogenase of *A. aerogenes* oxidized the L(+) hydroxyl group [L(+) dehydrogenase] to yield D(—)-AMC (Table I, column 4, and Fig. 1). Since *Ps. hydrophila* also oxidized *meso*-2,3-BD to D(—)-AMC (Table I, column 4), it also contains an L(+) dehydrogenase.

B. subtilis oxidized *meso*-2,3-BD to AMC with a low specific rotation (Table I,

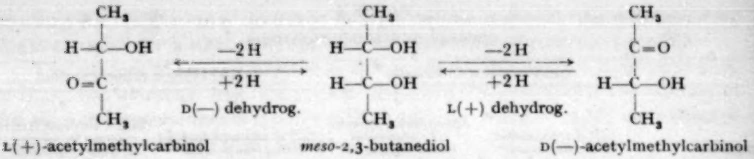

Fig. 1. Action of D(—)- and L(+)-2,3-butanediol dehydrogenases on *meso*-2,3-butanediol.

column 4). This has been explained by the demonstration of the D(—) and L(+) dehydrogenases in this bacterium. Thus, a freshly prepared crude sonic extract of *B. subtilis* cells oxidized both D(—)- and *meso*-2,3-BD. After this enzyme preparation had been stored at 4° for 6 days it still oxidized *meso*-2,3-BD, but was inactive when D(—)-2,3-BD was used as the substrate. An aliquot of the same enzyme preparation which had been kept at — 20° for the same period of time readily oxidized both the D(—)- and *meso*-2,3-BD.

The rates of oxidation of the isomers of 2,3-BD were determined manometrically. The results of these experiments are shown in Table II. The 10 min readings are initial rates. The 180 or 210 min readings were measured at a time when no further oxygen uptake was noticeable except in the one instance discussed below. These readings are to be compared with the theoretical values, given in the adjacent column, for complete oxidation to AMC.

The inability of *A. aerogenes* to oxidize D(—)-2,3-BD (Table II, column 3) is in agreement with the experiments involving DPN reduction (Table III) and suggests that this organism contains only an L(+) dehydrogenase. The addition of D(—)-2,3-BD

TABLE II

OXIDATION OF VARIOUS ISOMERS OF 2,3-BUTANEDIOL TO ACETYLMETHYLCARBINOL

The figures are μmoles of oxygen taken up by acetone-dried cell suspensions. Endogenous respiration was negligible.

Organism	*Time (min)*	*Isomer of 2,3-butanediol oxidised*							
		1		*2*		*3*		*4*	
		meso*		*Mixture* L(+) *and* meso**		D(—)***		*Mixture* D(—) *and* meso §	
		Exptl.	*Theor.*	*Exptl.*	*Theor.*	*Exptl.*	*Theor.*	*Exptl.*	*Theor.*
A. aerogenes	10	2.07	—	2.20	—	0.71	—	2.08	—
A. aerogenes	210	10.50	13.0	12.08	13.0	1.01	13.0	11.46	26.0
Ps. hydrophila	10	2.63	—	3.35	—	1.06	—	2.02	—
Ps. hydrophila	180	13.61	13.0	13.78	13.0	1.33	13.0	13.02	26.0
B. subtilis	10	0.65	—	0.75	—	1.06	—	1.58	—
B. subtilis	180	11.60	13.0	13.91	13.0	13.9	13.0	20.78	26.0
B. polymyxa	10	0.5	—	0.15	—	1.5	—	1.2	—
B. polymyxa	210	3.03	13.0	2.9	13.0	13.2	13.0	15.18	26.0

* 26 μmoles *meso*-2,3-BD.

** 26 μmoles L(+) and *meso* mixture of 2,3-BD obtained from the growth medium of *Ps. hydrophila* as described in METHODS.

*** 26 μmoles D(—)-2,3-BD obtained from growth medium of *B. polymyxa* as described in METHODS.

§ 26 μmoles D(—)-2,3-BD plus 26 μmoles *meso*-2,3-BD.

TABLE III

ACTIVITIES OF 2,3-BUTANEDIOL DEHYDROGENASES WITH VARIOUS ISOMERS OF 2,3-BUTANEDIOL

The rates were determined by measuring the reduction of DPN^+ in the presence of each substrate. Each cuvette contained sonic extract, 100 µmoles potassium phosphate buffer, pH 8.1, 0.4 mg DPN^+ and 26 µmoles of 2,3-BD. Total volume, 3 ml. The figures are the changes in optical density at 340 mµ from 0.5 to 2.0 min after addition of substrate.

Origin of enzyme	*Isomers of 2,3-butanediol*		
	meso	*Mixture of* L(+) *and* meso*	D(—)
A. aerogenes	0.120	0.144	0.036
Ps. hydrophila	0.114	0.150	0.045
B. polymyxa	0.043	0.043	0.080
B. subtilis	0.025	0.023	0.044

* The mixture of L(+) and *meso* isomers of 2,3-BD obtained from growth medium of *Ps. hydrophila* as described in METHODS.

did not inhibit the rate of oxidation of the *meso* isomer by *A. aerogenes* (Table II, columns 1 and 4). Similar results were obtained with *Ps. hydrophila* which also contains an L(+) dehydrogenase.

The ability of *B. subtilis* to oxidize all three isomers of 2,3-BD (Table II) is further evidence for the presence in this organism of both the D(—) and L(+) dehydrogenases.

Although *B. polymyxa* contains a D(—) dehydrogenase, the samples containing *meso*-2,3-BD were oxidized at rates considerably lower than the corresponding rate with D(—)-2,3-BD (Table II, columns 1 and 3). At 210 min the slow oxidation of *meso*-2,3-BD had not yet leveled off. Stanier and Fratkin[5] reported a similar result for *B. polymyxa*. Although *meso*-2,3-BD contains one hydroxyl group in the D(—) configuration, perhaps the configuration of the asymmetric carbon atom which is not oxidized [L(+)] is a factor that determines the activity of the D(—) dehydrogenase of *B. polymyxa*.

Cell suspensions of *A. aerogenes* and *Ps. hydrophila*, which contain L(+) dehydrogenases, showed a higher initial rate of oxygen uptake (10 min value) on the mixture of the L(+) and *meso* isomers of 2,3-BD than was obtained on pure *meso*-2,3-BD (Table II, columns 1 and 2). The initial rates of DPNH formation catalyzed by enzyme preparations of *A. aerogenes* and *Ps. hydrophila* were significantly greater in the presence of the mixture of L(+) and *meso* isomers of 2,3-BD than in the presence of *meso*-2,3-BD (Table III). Unfortunately, pure L(+)-2,3-BD was not available to test the relative rates of oxidation of the L(+) and the *meso* isomers of 2,3-BD in the presence of an L(+) dehydrogenase.

Evidence for acetylmethylcarbinol racemase

The data given for *Ps. hydrophila* in Table I, columns 3 and 4, show that AMC produced from pyruvate had a much lower specific rotation than AMC produced by the oxidation of *meso*-2,3-BD. This anomaly was explained by showing that *Ps. hydrophila* contained an AMC racemase. Incubation of an acetone-dried cell suspension of *Ps. hydrophila* with optically active AMC for 30 min resulted in a change from $[\alpha]_D^{25}$ $-95°$ ($c = 0.09$, in H_2O) to $[\alpha]_D^{25}$ $-53.5°$ ($c = 0.091$, in H_2O). A control experiment in which boiled cells were incubated with an aliquot of the optically active

AMC under the same conditions led to recovery of AMC which had an $[\alpha]_D^{25}$ $-90.7°$ ($c = 0.14$, in H_2O).

The fact that the specific rotation of AMC produced from the oxidation of *meso*-2,3-BD by *Ps. hydrophila* (Table I, column 4) was not low may be explained by the fact that AMC thus formed was trapped as the oxime in this experiment and was probably unavailable as a substrate for the racemase.

Stereoisomeric specificities of other 2,3-butanediol dehydrogenases

Bacteria that carry out the butanediol fermentation of sugars all contain 2,3-BD dehydrogenases. It has been shown that certain aerobic micrococci, which have been grown with *meso*-2,3-BD or *racemic* AMC as the sole source of carbon and energy, contain 2,3-BD dehydrogenases[2,4]. One of these organisms, *Micrococcus ureae*, produces 2,3-BD dehydrogenases when grown on sodium acetate, sodium lactate, *racemic* AMC, or *meso*-2,3-BD. Enzyme preparations from *M. ureae* that had been grown on *meso*-2,3-BD, sodium lactate, or sodium acetate were each tested for stereoisomeric specificity of the corresponding 2,3-BD dehydrogenase by determining the specific rotation of the AMC produced by oxidation of *meso*-2,3-BD. In each case L(+)-AMC was obtained, indicating that these enzymes were all D(—) dehydrogenases. (Fig. 1).

Some strains of *A. aerogenes* can grow with *racemic* AMC as the sole source of carbon and energy[4]. *A. aerogenes* grown on AMC contains a 2,3-BD dehydrogenase that has a different substrate specificity from the 2,3-BD dehydrogenase of *A. aerogenes* grown on glucose[4]. A comparison of the stereoisomeric specificities of these two dehydrogenases revealed that *A. aerogenes* grown on AMC contained a D(—)-2,3-BD dehydrogenase while glucose-grown cells contained an L(+)-2,3-BD dehydrogenase.

DISCUSSION

The data presented in this paper make it possible to explain the occurrence of the three isomers of 2,3-BD as end products of the fermentation of sugars by bacteria. As outlined in Fig. 2, pyruvate, produced from glucose during fermentation, is largely converted to D(—)-AMC which is a hydrogen acceptor and is reduced to 2,3-BD. *B. polymyxa* contains a stereoisomerically specific D(—)-2,3-BD dehydrogenase that catalyzes the reduction of the carbonyl group of D(—)-AMC to a hydroxyl group in the D(—) configuration to yield D(—)-2,3-BD (Fig. 2). The stereoisomerically specific L(+)-2,3-BD dehydrogenases in *A. aerogenes* and *Ps. hydrophila* catalyze the reduction of the carbonyl group of D(—)-AMC to a hydroxyl group in the L(+) configuration to yield *meso*-2,3-BD, which contains hydroxyl groups in both the L(+) and D(—) configurations (Fig. 2).

Ps. hydrophila contains an AMC racemase that catalyzes the conversion of some D(—)-AMC, formed from pyruvate, to L(+)-AMC (Fig. 2). Reduction of the carbonyl group of L(+)-AMC, catalyzed by the L(+) dehydrogenase in *Ps. hydrophila*, yields L(+)-2,3-BD. Thus, the L(+) dehydrogenase acting in combination with AMC racemase can explain the production of a mixture of L(+) and *meso* isomers of 2,3-BD found after the fermentation of sugars by *Ps. hydrophila*.

Presumably, the formation of some L(+)-2,3-BD by various strains of *A. aerogenes* is also due to the presence of an AMC racemase in these strains. We have been unable

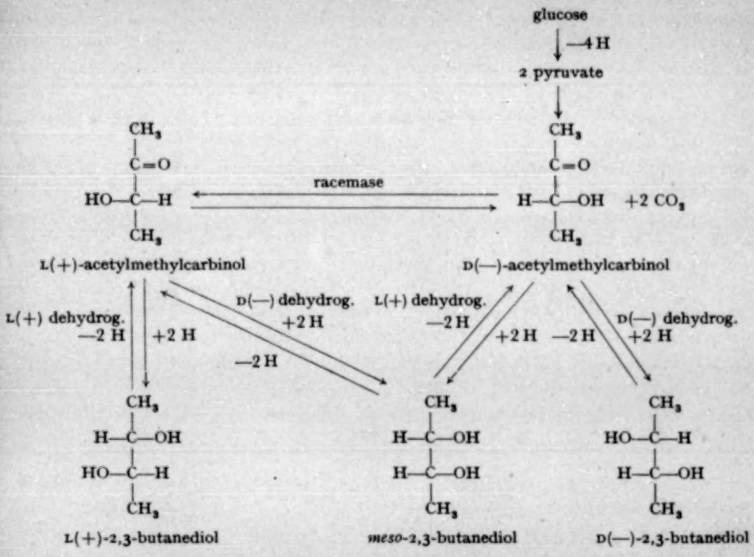
glucose
—4 H
2 pyruvate
racemase
+2 CO₂
L(+)-acetylmethylcarbinol
D(—)-acetylmethylcarbinol
L(+) dehydrog.
—2 H
+2 H
D(—) dehydrog.
+2 H
—2 H
L(+) dehydrog.
—2 H
+2 H
—2 H
D(—) dehydrog.
+2 H
L(+)-2,3-butanediol
meso-2,3-butanediol
D(—)-2,3-butanediol

Fig. 2. Formation of the three isomers of 2,3-butanediol from glucose.

to demonstrate an AMC racemase in the acetone-dried preparations of *A. aerogenes* studied in this laboratory. It is possible that extracts prepared by other procedures may be shown to contain an AMC racemase.

AMC produced from pyruvate by *B. subtilis* had a somewhat lower specific rotation than AMC produced by *B. polymyxa* or *A. aerogenes* (Table I, column 3) and it is possible that the *B. subtilis* preparation also contained an AMC racemase.

The finding that *B. subtilis* contains both a D(—) and an L(+) 2,3-BD dehydrogenase makes it possible to explain the mixture of D(—) and *meso* isomers of 2,3-BD found after fermentation of glucose (Table I, column 1). The carbonyl group of D(—)-AMC is partly reduced to a hydroxyl group in the D(—) configuration by the D(—) dehydrogenase to yield D(—)-2,3-BD. The L(+) dehydrogenase reduces the carbonyl group of D(—)-AMC to a hydroxyl group in the L(+) configuration to yield *meso*-2,3-BD (Fig. 2).

The presence of both a D(—) and an L(+) dehydrogenase and an AMC racemase in one organism can account for the formation of some *racemic* 2,3-BD. *Racemic* 2,3-BD production has been reported for *A. aerogenes*[17] and *Ps. hydrophila*[18]. The reduction of D(—)-AMC catalyzed by the D(—) dehydrogenase will produce D(—)-2,3-BD while the reduction of L(+)-AMC, formed from D(—)-AMC by the racemase, will yield L(+)-2,3-BD upon reduction by the L(+) dehydrogenase.

Alternatively, in the absence of an AMC racemase, the presence of the two stereoisomerically specific dehydrogenases is sufficient to account for the formation of some *racemic* 2,3-BD. Reduction of the naturally occurring D(—)-AMC by both dehydrogenases would yield D(—)- and *meso*-2,3-BD [D(—) and L(+) dehydrogenases respectively] (Fig. 2). Since these reactions are reversible, oxidation of *meso*-2,3-BD by the D(—) dehydrogenase would yield L(+)-AMC which could then be reduced to L(+)-2,3-BD by the L(+) dehydrogenase (Figs. 1 and 2).

The specific rotations of AMC produced by the oxidation of *meso*-2,3-BD (Table I, column 4) indicate that *A. aerogenes* and *Ps. hydrophila* contain only L(+) dehydrogenases and therefore should not be able to produce *racemic* 2,3-BD. It is possible that less drastic means of preparing cell-free extracts might reveal the presence of both D(—) and L(+) dehydrogenases in some other organism, since the D(—) dehydrogenase of *B. subtilis* is much more labile than the L(+) dehydrogenase. However, Stanier and Fratkin[5], working with freshly harvested, resting cell suspensions of *A. aerogenes* and *Ps. hydrophila*, could not demonstrate oxidation of D(—)-2,3-BD. *Meso*-2,3-BD was oxidized by resting cell suspensions of both organisms. It is possible that either some strains of *A. aerogenes* and *Ps. hydrophila* contain only L(+) dehydrogenases or that permeability barriers do not permit D(—)-2,3-BD to come in contact with D(—) dehydrogenases located within the cells. Since *B. subtilis* contains D(—)- and L(+)-2,3-BD dehydrogenases, and possibly an AMC racemase, it is possible that some of the optically inactive 2,3-BD formed during the fermentation of sugars may be *racemic* 2,3-BD.

Stanier and Adams[19] reported that *Ps. hydrophila* (*Aeromonas hydrophila*) produced a mixture of D(—)- and *meso*-2,3-BD, ($[\alpha]_D$ —0.97°). Working with a different strain of *Ps. hydrophila* we found that a mixture of L(+)- and *meso*-2,3-BD, $[\alpha]_D^{25}$ +0.94° ($c = 65.3$, in H_2O), was formed during the fermentation of glucose. If, as Stanier and Adams[19] reported, D(—)-2,3-BD had been formed during the fermentation of sugars, the same organism should have been able to oxidize the D(—) isomer of 2,3-BD. However, Stanier and Fratkin[5], using the same strain of *Ps. hydrophila* as used by Stanier and Adams[19], demonstrated that a resting cell suspension of this organism could not oxidize D(—)-2,3-BD.

Using a third strain of *Ps. hydrophila*, Murphy, Watson, Muirhead and Barnwell[18] showed that 2,3-BD formed by the fermentation of sugars consisted of a mixture containing 50% *racemic*, 48% *meso* and 2% D(—) isomers of 2,3-BD. This strain of *Ps. hydrophila* must contain both D(—) and L(+) dehydrogenases and probably an AMC racemase. The optical rotation of the 2,3-BD formed would depend upon the relative concentrations of the different enzymes.

It has been shown that other microorganisms contain stereoisomerically specific 2,3-BD dehydrogenases. Aubert and Gavard[2] demonstrated that oxidation of *meso*-2,3-BD by a cell-free extract of *Neisseria winogradskyi*, which had been grown on *meso*-2,3-BD as a source of carbon and energy, resulted in the formation of L(+)-AMC. This cell-free extract also oxidized D(—)-2,3-BD to D(—)-AMC. These results indicate the presence of a D(—) dehydrogenase. The oxidation of D(—)- and *meso*-2,3-BD, but not L(+)-2,3-BD (see ref. [1,6]), by various strains of *Acetobacter* demonstrates that *Acetobacters* also contain D(—)-2,3-BD dehydrogenases.

During a normal yeast fermentation a small quantity of 2,3-BD, ($[\alpha]_D$ —8.6°), is formed[20]. Since AMC produced by yeast is predominantly the D(—) isomer[21], reduction of this isomer of AMC by a D(—) dehydrogenase can explain the specific rotation obtained. The reduction of *racemic* AMC by fermenting yeast has yielded 2,3-BD with an $[\alpha]_D$ —5.5° (see ref. [22]), a finding consistent with the action of a

D(—) dehydrogenase. Reduction of D(—)-AMC by the D(—) dehydrogenase would yield D(—)-2,3-BD. The optically inactive *meso* isomer of 2,3-BD would result from the reduction of L(+)-AMC by the D(—) dehydrogenase. The reduction of diacetyl by fermenting yeast gave 2,3-BD with an $[\alpha]_D$ —2.4° (see ref. [23]), a result which suggests that the initial reduction of diacetyl to AMC may also be stereoisomerically specific, with the L(+) isomer of AMC being formed predominantly. Reduction of AMC, which is chiefly the L(+) isomer, would result in a higher concentration of optically inactive *meso*-2,3-BD (and a correspondingly lower concentration of D(—)-2,3-BD) than was obtained when *racemic* AMC was reduced by fermenting yeast.

Sebek and Randles[24] have reported that *Pseudomonas fluorescens* can be grown with any of the three isomers of 2,3-BD as the sole source of carbon and energy. The stereoisomeric specificities of the 2,3-BD dehydrogenases present in *Ps. fluorescens* when grown with different isomers of 2,3-BD were not studied.

As shown in Table III, the configuration of the asymmetric carbon atom of 2,3-BD which is not oxidized affects the rate of oxidation of 2,3-BD. In each case studied the optically active isomer was oxidized more rapidly than *meso*-2,3-BD. Studies by Aubert and Gavard[2] with a 2,3-BD dehydrogenase obtained from *N. winogradskyi* grown with *meso*-2,3-BD, rather than sugars, as carbon source showed that *meso*-2,3-BD was oxidized at a more rapid rate than was the D(—)-2,3-BD.

It is interesting to observe that members of the group of enzymes catalyzing the oxidation of 2,3-BD differ not only in stereoisomeric specificity but also in substrate specificity and substrate affinity[4]. Thus, the 2,3-BD dehydrogenases seem to provide promising material for studies of the mode of action of dehydrogenases.

ACKNOWLEDGEMENT

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PSEUDOUROPORPHYRIN, AND ENZYMIC AND NON-ENZYMIC PORPHYRIN FORMATION *IN VITRO*

J. E. FALK* AND E. I. B. DRESEL**

Department of Chemical Pathology, University College Hospital Medical School, London (Great Britain)

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SUMMARY

Much information can be derived from paper-chromatographic identification of the products in studies of porphyrin biosynthesis.

It is suggested that pseudouroporphyrin may be a derivative of an as yet unidentified intermediate in the biosynthetic pathway.

At physiological pH and temperature, uroporphyrin III can be formed non-enzymically.

Preferential catalysis of the conversion of porphobilinogen to uroporphyrin I appears to occur at the external cell wall of erythrocytes.

Particle-bound enzymes are required in the biosynthesis at stages between glycine and δ-aminolaevulinic acid, and between coproporphyrinogen and protoporphyrin. Oxygen is required at the same two stages.

It is these particle-bound oxidative enzyme systems, present in mammalian erythrocyte precursors, which are lost when these cells mature.

INTRODUCTION

It is now known that the early steps in porphyrin biosynthesis involve the condensation of glycine and succinate to yield the specific porphyrin precursor, δ-aminolaevulinic acid (ALA)[1,25], two molecules of which condense to form the monopyrrole porphobilinogen (PBG)[2]. Both ALA and PBG serve as useful substrates for studies of porphyrin formation *in vitro*[3–7]. In this paper, the types of porphyrins formed from these substrates in various *in vitro* conditions are discussed.

Particular reference is made to the porphyrin isomers formed. Uroporphyrin III and coproporphyrin III are of the "natural" isomeric series, to which belong the chlorophylls and the haems of all the haemoproteins of known structure. Uroporphyrin I and coproporphyrin I occur naturally, but appear in significant amounts in pathological conditions only. Strangely, chicken erythrocytes possess an enzyme system capable of converting uroporphyrin I to coproporphyrin I[7].

Because it is desired here to make qualitative comparisons of the patterns of formation of the various porphyrins under different conditions, and because of the

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[*] Present address: Division of Plant Industry, C.S.I.R.O., Canberra, Australia.
[**] Present address: P.O. Box 7587, Johannesburg, South Africa.

diverse character of the incubation mixtures, it is not considered useful to quote quantitative data. Such data have been given for incubations of intact chicken erythrocytes[4], and preparations from these[5]; the remainder of the results quoted are from unpublished experiments.

METHODS

The porphyrins were isolated and determined and paper chromatographic analyses performed as described by FALK, DRESEL, BENSON AND KNIGHT[6]. In these analyses, copro- and protoporphyrin are isolated and determined quantitatively, as is the total "uroporphyrin". The latter determination includes uroporphyrins I and III, and pseudouroporphyrin. Estimates of the relative proportions of these three porphyrins are then made from paper chromatograms.

RESULTS

In Table I are summarized some conditions leading to the formation of uroporphyrin III, coproporphyrin III and protoporphyrin. It will be observed that pseudouroporphyrin is always formed concurrently.

TABLE I

CONDITIONS FOR THE FORMATION *in vitro* OF UROPORPHYRIN III AND PSEUDOUROPORPHYRIN

Incubation was at 37° and pH 7.3. Blanks in the table indicate no detectable formation.

Substrate	*System*	*Porphyrins formed*				
		Uro-III	*ψ-Uro*	*Uro-I*	*Corpo-III*	*Prote*
1. PBG, ALA	Chicken erythrocyte haemolysate	+	+		+	+++
2. ALA	Washed intact chicken erythrocytes	+	+			+++
3. ALA	Whole normal rabbit blood	++	++		+	Trace
4. ALA	Whole normal human blood	+	+		+++	Trace
5. PBG	Supernatant, chick erythrocyte haemolysate	++	++		++	Trace

Pseudouroporphyrin[6,8] appears, in amounts about equivalent to uroporphyrin III, when either ALA or PBG is exposed to enzymes on the biosynthetic pathway leading to copro- and protoporphyrins. The formation of significant amounts of pseudouroporphyrin was not observed under conditions where enzymes were presumably not involved (Tables II and III) though uroporphyrin III is readily formed under such conditons (Table III). Pseudouroporphyrin is not one of the four known uroporphyrin isomers, but spectroscopically, in its solubility properties, and in its behaviour on paper chromatograms it is very like a uroporphyrin[6]. On decarboxylation it yields coproporphyrin III only[8]. Its properties are consistent with the suggestion that it may be a simple derivative of uroporphyrin III, and it could conceivably be an oxidation product of some kind of cofactor-complex of uroporphyrinogen III, which is apparently[9-11] a true precursor of protoporphyrin. Evidence for a cofactor requirement at this region of the biosynthetic chain has been found recently; thus the formation from PBG of uro-, copro- and protoporphyrins, in a brei of ineffective root nodules of soy bean, does not occur unless boiled yeast juice, or a boiled extract of effective nodules, is added[12]. Further, a heat stable factor necessary for the formation of coproporphyrin from PBG has been found[13] in extracts of *Rhodopseudomonas spheroides*. Boiled yeast juice stimulated 6-fold the formation of haem from glycine by haemolysed chicken erythrocytes[14], but this was probably due to stimulation of ALA formation (see also ref. [15]).

As shown in Table I, chicken blood, normal human blood and normal rabbit blood all convert ALA to uroporphyrin III, pseudouroporphyrin and coproporphyrin, but, unlike avian blood, both human and rabbit erythrocytes lack the enzyme system for the formation of protoporphyrin. This enzyme system is particle-bound, and may be supplied in the form of rat-liver mitochondria; thus a particle-free supernatant from haemolysed chicken erythrocytes makes uro- and coproporphyrins only from ALA or PBG, but on addition of rat liver mitochrondria protoporphyrin is formed[5,16]. While avian erythrocytes and mammalian reticulocytes, which contain mitochondrial processes[17] and respire actively, are able to carry out all the steps leading from glycine to protoporphyrin, the non-nucleated, slowly respiring mammalian erythrocytes make no porphyrins from glycine. No doubt this is due to their lack of the enzymes required for the formation of succinyl-coenzyme A, the precursor of ALA[18–21], and for the condensation of succinyl-coenzyme A with glycine. The latter enzymes have been shown to be particle-bound[20].

TABLE II

CONDITIONS FOR THE FORMATION *in vitro* OF UROPORPHYRIN I AS MAIN PRODUCT

Substrate	*System*	*Porphyrins formed*				
		Uro-III	*ψ-Uro*	*Uro-I*	*Copro-III*	*Proto*
1. PBG	Whole chicken blood	+	Trace	+++	Trace	Trace
2. PBG	Washed intact chicken erythrocytes	+	Trace	+++	Trace	Trace
3. PBG	Whole normal rabbit blood	+	Trace	+++	Trace	Trace
4. PBG	Whole normal human blood	+		+++		
5. PBG	Water, pH 10*	+		+++		

* Several days at 20° (see ref. 24). All other incubations were at 37° and pH 7.3.

PBG behaves quite differently from ALA on incubation with chicken, human or rabbit blood which has not been haemolysed (Table II). The majority of the porphyrin formed is uroporphyrin I, accompanied by some uroporphyrin III and traces only of other porphyrins. These results indicate that PBG cannot penetrate the erythrocytes freely and that the conversion to uroporphyrin I may occur outside the cells. The traces of pseudouroporphyrin, coproporphyrin and protoporphyrin formed apparently reflect slight penetration of the cells by the PBG. When the cells were boiled prior to incubation, uroporphyrin I formation was greatly reduced, and it appears that perhaps a surface catalysis at the cell wall was operating. The cell walls were still present, though ruptured, in the haemolysate (Table I, No. 1), but here incubation with PBG led to no uroporphyrin I; this was perhaps due to exhaustion of the PBG by its more rapid enzymic conversion in this system to uro-III, pseudouro-, copro- and protoporphyrins.

TABLE III

CONDITIONS FOR THE FORMATION *in vitro* OF UROPORPHYRIN III AS MAIN PRODUCT

Substrate	*System*	*Porphyrins formed*				
		Uro-III	*φ-Uro*	*Uro-I*	*Copro-III*	*Proto*
PBG	Dilute chicken serum*	+++		Trace		
PBG	Ditto boiled (pH 7.3)	+++		Trace		
PBG	Whole chicken serum with 0.01 *M* Versene (pH 7.3)	+++		Trace		
PBG	0.2 *N* HCl**	+++				
PBG	Acetate buffer** pH 4.2	+++				
PBG	Normal urine** pH 4.2	+++				
PBG	Acute porphyria urine** pH 4.2	+++	Trace	+	Trace	

* Diluted with saline.
** Heated at 93° for 20 min; all others incubated at 37° for 18 h.

In Table III, a variety of conditions is shown in which PBG is converted to uroporphyrin III only. These are all obviously non-enzymic conditions; they may be considered, indeed, as aqueous solutions of pH less than 8. It is interesting that while uroporphyrin III was the main product on incubation of PBG with serum, whether this had been boiled or not (Table III), the presence under otherwise identical conditions of intact chicken, human or rabbit erythrocytes, impermeable to PBG, determined that uroporphyrin I was the main product (Table II).

There is little doubt that pseudouroporphyrin is a real entity[8], and not a chromatographic artifact, and all the evidence of its occurrence, described above, suggests that it may have biosynthetic significance. Its formation is not restricted to erythrocytes; it has recently been found, along with uro- III, corpro- III and protoporphyrins, after incubation of a brei of young oat leaves with PBG (*cf.* ref. [12]). It has not been reported in several recent studies[11, 22, 23] where its formation might have been expected. It is possible that it is a side-product from uroporphyrinogen III, and that in the bacteria[22] and in the isolated enzyme systems[11, 23], the reactions leading to its formation were absent. It is equally possible that in these systems its conversion (presumably *via* its leuco-compound) to further products occurred too quickly to allow it to accumulate.

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SÉPARATION ET CARACTÉRISATION DE LA N-ÉTHYL-γ-GLUTAMINE À PARTIR DE *XEROCOMUS BADIUS*

J. CASIMIR, J. JADOT ET M. RENARD
Laboratoires de Chimie Générale et Organique, Institut Agronomique de l'Etat à Gembloux et Université de Liège (Belgique)

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SUMMARY

Separation and identification of N-ethyl-γ-glutamine from Xerocomus badius

A derivative of glutamic acid has been found in the non protein nitrogen fraction of the mushroom *Xerocomus badius*.

The compound was isolated by paper partition chromatography and was shown to be N-ethyl-γ-glutamine.

The structure was confirmed by comparison with the synthetic compound.

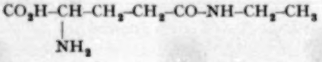
Different steps involved in the possible biogenesis of N-ethyl-γ-glutamine have been discussed.

INTRODUCTION

Au cours de ces dernières années, le développement considérable des techniques chromatographiques sur papier et sur colonnes de résines échangeuses d'ions a permis la découverte de nombreux acides aminés nouveaux dans la fraction soluble dans l'alcool des organismes végétaux. Un grand nombre de ces substances sont des dérivés de l'acide glutamique; certains ont été découverts par Virtanen *et al.*: l'acide γ-hydroxyglutamique dans *Phlox decussata*[1], l'acide $\beta\gamma$-dihydroxyglutamique dans les graines de *Lepidium sativum* et dans les parties vertes de *Rheum rhaponticum*[2,3], l'acide γ-méthyl glutamique et l'acide γ-méthyl-γ-hydroxyglutamique dans *Phyllitis scolopendrium*[4,5]. Signalons la présence de l'acide γ-méthylèneglutamique et de l'amide correspondante décrite par Done et Fowden[6] dans *Arachis hypogaea* et par Zacharius, Pollard et Steward[7] dans la tulipe. Parmi les peptides dérivés de l'acide glutamique signalons aussi la découverte de la γ-glutamylalanine et de l'acide γ-glutamyl-valyl glutamique respectivement dans le pois en germination et dans *Juncus conglomeratus* par VIRTANEN *et al.*[8,9] et celle de la γ-glutamyl-S-méthyl cystéine par ZACHARIUS, MORRIS ET THOMPSON[10] dans *Phaseolus vulgaris*.

La présente publication décrit l'isolement et la détermination de la structure d'un acide aminé, dérivé de l'acide glutamique, à partir d'un champignon de la famille des Boletacées: *Xerocomus badius* (Fr.)*.

Cette substance, la N-éthyl-γ-glutamine:



$$\underset{\displaystyle NH_2}{CO_2H\text{-}\underset{|}{CH}}\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}CH_3$$

existe en quantité importante dans ce champignon. L'acide aminé naturel et la N-éthyl-γ-glutamine préparée par synthèse possèdent des propriétés physiques et chimiques identiques.

MÉTHODES ET RÉSULTATS

Techniques chromatographiques

L'analyse bidimensionnelle a été réalisée à l'aide de papier Schleicher et Schüll No. 2043b avec le choix de solvants suivants: (a) *n*-butanol–acide formique–eau (75:15:10); phénol saturé par un tampon phosphate–acide citrique à pH = 4.2[11]. (b) *n*-butanol–acide acétique–eau (63:27:10); phénol saturé d'eau en présence d'ammoniac[12].

La chromatoélectrorhéophorèse sur papier a été effectuée avec le solvant *n*-butanol–acide formique dans le sens de la chromatographie et avec une solution d'acide acétique *N* dans la direction de l'électrophorèse[13].

Les acides aminés ont été révélé à l'aide d'une solution de ninhydrine 0.25 % dans l'éthanol 95 %.

Le papier Whatman No. 3 a été utilisé pour la préparation chromatographique de la substance.

L'étude de la distribution des acides aminés libres dans la fraction soluble dans l'éthanol 80 % des bolets, nous a permis de mettre en évidence sur les chromatogrammes A et B de *Xerocomus badius* une tache nouvelle; cette tache de coloration violette intense avec la ninhydrine se trouve à proximité du front du phénol et au dessus de la valine. Un spot correspondant se retrouve à la chromatoélectrorhéophorèse à gauche de l'alanine et en dessous de l'acide glutamique et de la thréonine (spot M des Figs. 1 et 2).

Le Tableau I indique les valeurs des R_F de la tache nouvelle pour les différents solvants utilisés.

Ces propriétés chromatographiques et la sensibilité particulière de la substance à l'hydrolyse par les acides et les bases même dilués nous ont permis de considérer le composé M comme un constituant peu commun des organismes végétaux et de mettre au point une technique d'isolement**.

Isolement de la substance

Les carpophores frais (2,140 kg) ont été pulvérisés dans un Waring blendor en présence de 8.5 l d'éthanol 95 %, de façon à obtenir une concentration approximative

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* L'identification des récoltes est due au Dr. P. HEINEMANN à qui nous adressons nos sincères remerciements.

** Il se peut qu'il soit identique à la "théanine" isolée des feuilles de thé par Y. SAKATO[22].

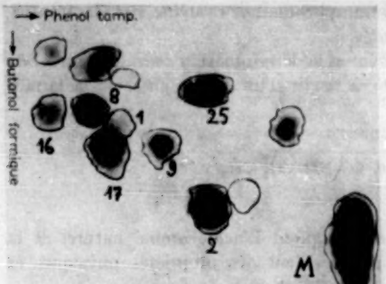


Fig. 1. Chromatogramme bidimensionnel de l'extrait de *Xerocomus badius* (solvant (a)). 16, acide aspartique; 17, acide glutamique; 8, sérine; 1, glycine; 9, thréonine; 2, alanine; 3, valine; 5, leucines; 25, glutamine; M, acide aminé inconnu.

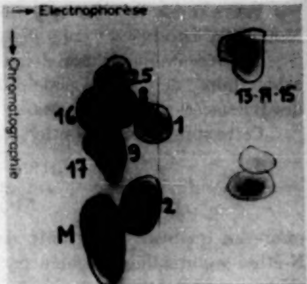


Fig. 2. Chromatoélectrorhéophorèse de l'extrait de *Xerocomus badius*. Numérotation comme Fig. 1.

TABLEAU I

COMPARAISON DES R_F DU SPOT M AVEC CEUX D'AUTRES ACIDES AMINÉS

Acides aminés	*Phénol-tampon.*	*Phénol-NH$_3$*	*BuOH-HCO$_2$H*	*BuOH-CH$_3$CO$_2$H*
M	0.81	0.81	0.38	0.32
Ac. glutamique	0.26	0.16	0.22	0.14
Alanine	0.47	0.51	0.32	0.21
Valine	0.72	0.70	0.53	0.42
Leucine	0.81	0.76	0.66	0.57

de 80 % en éthanol dans l'extrait filtré. L'extrait a été ensuite concentré sous pression réduite et évaporé à sec. La tyrosine, la phénylalanine et les matières colorantes ont été éliminées par adsorption sur du charbon traité par de l'acide acétique selon la méthode de PARTRIDGE[14]. Le filtrat final a été passé sur une colonne d'Amberlite IR-120 sous la forme H^+ et après lavage de la colonne à l'eau, les acides aminés ont été élués par une solution de NH_3 N; l'éluat après concentration et dessiccation a donné un résidu de 5.2 g. La purification ultérieure de la substance a été achevée par chromatographie sur papier à l'aide de la technique décrite par FOWDEN[15]. Les feuilles de Whatman No. 3, après lavage avec les réactifs suivants: HCl 0.5 N; eau; NaOH 0.5 N; eau; alcool, ont été séchées à 40°. Le résidu précédent a été dissous dans 25 ml d'eau et appliqué en une ligne continue sur 16 feuilles du papier préparé. Après séparation chromatographique avec le solvant *n*-butanol–acide acétique–eau et séchage, les parties de chromatogramme contenant la substance M ont été localisées à l'aide de spots témoins et éluées à l'eau. L'éluat a été enfin concentré et évaporé à sec. La recristallisation du résidu dans le méthanol absolu à permis d'obtenir 430 mg de substance M sous forme de cristaux blancs et chromatographiquement pure.

La substance se dissout facilement dans l'eau, sa dissolution dans le méthanol est plus difficile.

Analyse élémentaire

Le produit, qui fond à 221°, donne à l'analyse: C: 48.27 %; H: 8.14 %; N (Kjeldahl): 16.15 %; N (Van Slyke): 15.07 %.

On peut déduire de ces résultats la formule brute: $C_7H_{14}O_3N_2$ qui donne les valeurs calculées: C: 48.26 %; H: 8.10 %; N: 16.08 %.

Détermination de l'équivalent-gramme

(a) Titration par l'acide perchlorique en milieu anhydre (acide acétique glacial): 7.85 mg de la substance ont été titrés par 1.81 ± 0.01 ml de $HClO_4$ 0.025 *N*, ce qui correspond à un équivalent-gramme de 173. (b) Dosage à la ninhydrine selon VAN SLYKE, MACFADYEN ET HAMILTON[16]. Une prise d'essai de 3.16 mg a été attaquée par la ninhydrine et le CO_2 dégagé absorbé par 3 ml de baryte correspondant à 11.54 ml de HCl 0.02 *N*. L'excès de baryte a nécessité 9.74 ± 0.02 ml et la prise d'essai 1.8 ml de HCl 0.02 *N* ce qui correspond à un équivalent-gramme de 175.

Détermination du poids moléculaire

La méthode de BARGER[17] donne 174, ce qui permet de conclure à l'identité des formules brute et moléculaire.

Hydrolyse de la substance

La substance a été hydrolysée pendant 20 h à 108°, d'une part en présence de HCl *N* et d'autre part en présence de HCl 6 *N*. Le résultat des deux hydrolyses a été identique, la tache correspondant à la substance a disparu des chromatogrammes avec apparition de deux spots nouveaux. Le 1er spot n'a pu être séparé de l'acide glutamique par chromatographie sur papier en utilisant les solvants décrits précédemment; de plus, ce composé a migré comme l'acide glutamique à l'électrorhéophorèse sur papier en présence d'un tampon phosphaté à pH = 5.6. Le second spot, de R_F semblables à ceux de l'éthylamine, a migré dans une direction identique à celle des substances basiques. Dans les mêmes conditions expérimentales, la substance non hydrolysée migre comme un acide aminé neutre.

L'hydrolyse alcaline du composé par la baryte saturée pendant un heure à 100° a scindé également la molécule en acide glutamique et en éthylamine; une très petite quantité de l'acide aminé est resté inchangée après l'hydrolyse.

Dosage de l'amine libérée par l'hydrolyse

5.195 mg de substance ont été chauffés à reflux avec 3 ml de HCl 6 *N* pendant 5 h. La solution a été alors transférée quantitativement dans un semi-micro appareil d'entraînement à la vapeur d'eau muni de deux boules de Kjeldahl, la première remplie de billes de verre et l'autre vide. La solution a été rendue basique, puis l'amine a été entraînée à la vapeur pendant 20 min. Le distillat a été recueilli dans 15 ml de HCl 0.01 *N*. L'excès de HCl a été titré par 12 ml de NaOH 0.01 *N*. L'amine correspondant à 3 ml de HCl 0.01 *N*, on peut donc considérer que la molécule contient 25.3 % d'éthylamine (calculé théoriquement: 25.28 %).

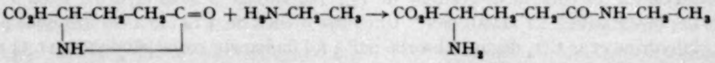
La solution du sel de l'amine a été fortement concentrée et le résidu donne un test de Rimini positif montrant la présence d'une amine primaire.

Dosage de l'acide glutamique après hydrolyse

L'acide glutamique a été déterminé par chromatographie sur papier avec le solvant *n*-butanol–acide formique–eau, révélation à l'aide du réactif de BARROLLIER[18] et colorimétrie du spot élué. Le résultat montre que la molécule de l'acide aminé est constitué par 75 % d'acide glutamique (calculé théoriquement 74.72 %).

Synthèse de la N-éthyl-γ-glutamine

La substance a été préparée selon la méthode de LICHTENSTEIN[19] par action d'une solution aqueuse d'éthylamine sur l'acide pyrrolidone 2 carboxylique 5; les conditions expérimentales ont été modifiées de façon à augmenter la vitesse de la réaction.



$$\mathrm{CO_2H{-}\underset{\underset{NH}{|}}{CH}{-}CH_2{-}CH_2{-}\underset{|}{C}{=}O + H_2N{-}CH_2{-}CH_3 \rightarrow CO_2H{-}\underset{\underset{NH_2}{|}}{CH}{-}CH_2{-}CH_2{-}CO{-}NH{-}CH_2{-}CH_3}$$

L'acide pyrrolidone 2 carboxylique 5 a été préparé selon la méthode de FISHER ET BOEHNER[20]. Une solution de 2.5 g de cette substance dans 12 ml d'eau a été introduite dans un tube en pyrex à paroi épaisse et refroidie à 0°; 12 ml d'une solution d'éthylamine à 50 % y ont été ajoutés et le tube scellé a été abandonné à l'étuve pendant 10 jours à 37°. Après ouverture du tube, la solution a été laissée pendant quelques heures à l'air libre puis placée dans un dessiccateur d'abord sous vide partiel puis sous vide poussé. Il s'est formé une masse cristalline qui a été reprise par l'éthanol absolu. Les cristaux séchés sous vide présentant un point de fusion de 200°, une double recristallisation dans le méthanol absolu a permis d'obtenir une préparation caractérisée par un point de fusion de 220°. (rendement en substance recristallisée: 33 %).

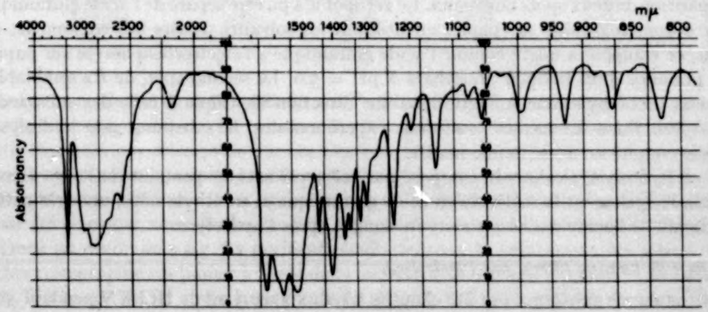


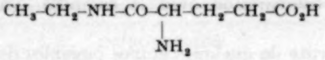
Fig. 3. Spectre infra-rouge de la N-éthyl-γ-glutamine naturelle.

Mélangée à la substance naturelle, la substance synthétique n'a pas provoqué d'abaissement de point de fusion. Ses R_F dans les différents solvants utilisés pour la chromatographie sur papier sont semblables.

CONCLUSIONS

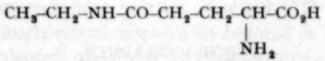
Sur la base des différents résultats expérimentaux obtenus, on peut conclure à l'identité du composé séparé avec la N-éthyl-γ-glutamine. La substance naturelle et le produit préparé par synthèse possèdent des propriétés chimiques et physiques identiques. Les résultats des hydrolyses acide et alcaline, les dosages d'acide glutamique et d'éthylamine sont tout à fait concordants et constituent la preuve de l'existence d'une liaison du type –NH–CO– entre l'acide glutamique et l'éthylamine. Les spectres infra-rouge des substances naturelle et synthétique en présence de KBr comme phase solide (concentration 1 mg pour 200 mg de KBr) sont absolument superposables comme le montre les Figs. 3 et 4.

La structure de la N-éthyl-γ-glutamine est semblable à celle d'un dipeptide avec le groupement glutamyle en position γ. Sachs et Brand[21] ont montré que les peptides de ce type avec un groupement aminé et un groupement carboxyle libre libèrent 2 moles d'azote aminé par mole de substance lors du dosage de Van Slyke à l'acide nitreux. Dans les mêmes conditions, les α-glutamyl peptides donnent une mole d'azote aminé par mole de substance. Le rapport de l'azote aminé à l'azote total étant de 0.92, on peut rejeter une formule du type



$$CH_3{-}CH_2{-}NH{-}CO{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}CH_2{-}CH_2{-}CO_2H$$

pour adopter la structure suivante:



$$CH_3{-}CH_2{-}NH{-}CO{-}CH_2{-}CH_2{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}CO_2H$$

ce que confirme d'ailleurs la synthèse.

Différentes hypothèses peuvent être envisagées au sujet de la biosynthèse de l'acide aminé. On pourrait le considérer comme un produit de décarboxylation de la γ-glutamylalanine; cependant ce peptide n'a pas été trouvé par chromatographie

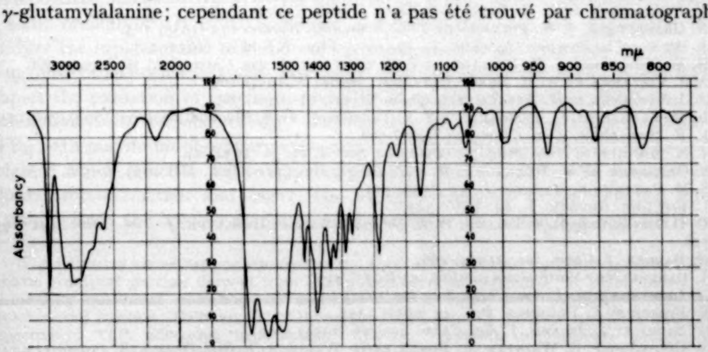


Fig. 4. Spectre infra-rouge de la N-éthyl-γ-glutamine synthétique.

sur papier de plusieurs extraits alcooliques de *Xerocomus badius*. De plus la décarboxylation dans les plantes de substances du type de l'acide glutamique se fait généralement aux dépens de la fonction acide voisine du groupement α aminé; c'est le cas de l'acide γ-aminobutyrique qui est beaucoup plus répandu dans le règne végétal que l'acide α-aminobutyrique.

Certains enzymes du type glutamotransférase, isolés de bactéries, sont capables de catalyser l'échange des groupes amidés de la glutamine ou de l'asparagine avec l'hydroxylamine. Waelsch *et al.*[22] ont ainsi préparé des acides hydroxamiques par action d'enzymes extraits de *Proteus vulgaris*; la synthèse de ces mêmes acides hydroxamiques est également possible à partir d'acide glutamique ou d'acide aspartique mais cette dernière réaction, qui est utilisée comme modèle pour la synthèse de la glutamine, nécessite la présence d'adénosine triphosphate et de magnésium. Il serait possible de considérer que la N-éthyl-γ-glutamine se forme par transfert du radical glutamyle sur l'éthylamine à l'intervention de semblables enzymes.

Nous remercions le centre interuniversitaire de chimie organique pour l'aide qu'il nous a apportée au cours de cette recherche.

RÉSUMÉ

On a mis en évidence la présence d'un dérivé de l'acide glutamique dans la fraction soluble dans l'alcool du champignon *Xerocomus badius*. La substance a été isolée par chromatographie de partition sur papier et il a été prouvé qu'il s'agissait de la N-éthyl-γ-glutamine. La comparaison avec le composé synthétique a confirmé la structure proposée.

Finalement, on discute de quelques étapes possibles de la biosynthèse de cette substance.

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STUDIES ON THE MECHANISM OF OXIDATIVE PHOSPHORYLATION

VI. A FACTOR FOR COUPLED OXIDATION IN THE ELECTRON TRANSPORT PARTICLE

ANTHONY W. LINNANE* AND EDWARD B. TITCHENER*

Institute for Enzyme Research, University of Wisconsin, Madison, Wisc. (U.S.A.)

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SUMMARY

A procedure is described for the separation of the electron transport particle, ETP_H, into two components, one a non-phosphorylating ETP_H, the other a soluble protein. From these in the presence of Mg^{++} the phosphorylating system can be re-constituted; the maximum P/O ratios are respectively 1.0 and 0.6 for DPNH and succinate as substrates. The non-phosphorylating ETP_H and the protein factor appear to be firmly bound together by Mg^{++}.

INTRODUCTION

In previous communications from this laboratory[1, 2] the preparation and properties of two types of electron transport particle, denoted ETP and ETP_H have been described. Beef-heart mitochondrial suspensions isolated in 0.25 *M* sucrose[1] contain mitochondria of varying degrees of damage and fragmentation which can be separated into a heavy mitochondrial fraction (H-BHM) and a light mitochondrial fraction (L-BHM)[3]. ETP is derived from fragments in the L-BHM[4], while the more intact mitochondrial forms in the H-BHM give rise to ETP_H after appropriate exposure to sonic oscillation[2]. It is characteristic of both of these particle types that they catalyze the rapid aerobic oxidation of succinate and DPNH and that they are free from most of the other enzymes associated with the citric acid cycle. While ETP_H couples the oxidation of succinate and DPNH to phosphorylation, ETP has little such activity. The presence of Mg^{++} was found to be essential for the preparation of ETP_H with maximum phosphorylative activity. Recently a modified ETP_H has been isolated[5] which is active with regard to phosphorylation only in the presence of a soluble protein fraction and Mg^{++}. The oxidative capacity of the modified ETP_H is unimpaired: the soluble component effects only the recoupling of DPNH and

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The following abbreviations are used in the text: ETP, electron transport particle; ETP_H, electron transport particle derived from heavy beef-heart mitochondria; H-BHM, heavy, fast sedimenting beef-heart mitochondrial fraction; L-BHM, light, slow sedimenting beef-heart mitochondrial fraction; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide respectively; ATP, adenosine triphosphate; ATP'ase, adenosine triphosphate phosphatase; EDTA, ethylene diamine tetraacetate pH 7.4; GSH, glutathione.

* Postdoctoral Trainees of the University of Wisconsin, Institute for Enzyme Research.

succinate oxidation to phosphorylation. ETP derived from L-BHM differs from modified ETP_H in that ETP cannot recouple succinate and DPNH oxidation to phosphorylation, even in the presence of the soluble protein and Mg^{++}.

This communications gives details of the preparation and some of the properties of the modified ETP_H and the soluble component. Evidence is also presented that the recombination of the soluble factor with the modified ETP_H requires the presence of Mg^{++}.

EXPERIMENTAL

Inorganic phosphate and protein were determined by the methods of FISKE AND SUBBAROW[6] and of GORNALL *et al.*[7] respectively. Reagent and chemical sources have been specified in previous publications from this laboratory[2].

Manometric measurements were made at 30° by conventional Warburg technique. The usual duration of experiments was 20–40 min. Oxygen uptake values were corrected for the blank values (in the absence of additional substrate) which were of the order of 1 μatom and were due to the presence of small amounts of succinate in the medium used for the storage of the modified ETP_H.

For the measurement of oxidative phosphorylation the following components were introduced into the main compartment of the manometer flask; ATP (5 μmoles), $MgCl_2$ (20 μmoles), potassium phosphate of pH 7.3 (20 μmoles), glucose (100 μmoles), sucrose (1 mmole), yeast hexokinase (0.5 mg) and 1–3 mg protein of the particle suspension in a final volume of 3.0 ml; 0.2 ml of 6 N KOH was placed in the center well. Where indicated succinate (40 μmoles) or a DPNH generating system was added. The DPNH generating system was made up of crystalline yeast alcohol dehydrogenase (0.3 mg) free from ammonium sulphate, ethanol (60 μmoles), semicarbazide (15 μmoles) and DPN (1 μmole). This complete DPNH generating system is denoted hereinafter as DPNH.

Preparation of modified ETP_H

H-BHM freshly isolated by the method of HATEFI *et al.*[3] are washed with 5–10 volumes of 0.001 M EDTA–0.25 M sucrose, then with 5–10 volumes of 0.25 M sucrose. The mitochondria are next aged at — 10° for at least 5 days with several freezings and thawings throughout that period. The aged H-BHM suspensions are diluted with sufficient 0.25 M sucrose to achieve a final protein concentration of about 20 mg/ml; EDTA is added to a final concentration of $2 \cdot 10^{-4}$ M; and the mixture is adjusted to pH 7.3. These suspensions (in 25-ml aliquots) are then treated for 60 sec in a 10 kc Raytheon sonic oscillator at an energy input of 1.0 A with the temperature maintained near 2°. The combined treated suspensions are centrifuged 5 min at 15,000 × g. The residue is discarded, and the supernatant fluid after decantation is centrifuged for 40 min at 100,000 × g. This final supernate is the source of the soluble component and it is set aside for subsequent fractionation. The sediment is collected, washed first with about 10 volumes of 0.25 M sucrose–0.001 M EDTA, pH 7.2, and then with about 10 volumes of 0.25 M sucrose. Finally the modified ETP_H is suspended in 0.25 M sucrose containing $MgCl_2$ (5 μmoles/ml), GSH (2 μmoles/ml), ATP (2 μmoles/ml) and succinate (4 μmoles/ml). The final protein concentration is adjusted to about 10 mg/ml and the preparation is stored at 1°.

The yield of modified ETP_H is considerably influenced by the pretreatment of the H-BHM. Whereas the yield of ETP_H from mitochondria which have been aged for 24 h and frozen and thawed several times, is about 5–10 % of the total protein, the yield of ETP_H from mitochondria aged 5–7 days with intermittent freezing and thawing is upwards of 50–70 % of the total protein.

Fractionation of "sonic" supernate for the phosphorylation factor

A flow sheet for the fractionation of the "sonic" supernate is shown in Fig. 1. The fractionation is complicated by several factors. Firstly, the unfractionated "sonic" supernate contains an inhibitor of phosphorylation; the addition of small amounts to the modified ETP_H induces phosphorylation, which reaches a maximum value (never exceeding a P/O ratio of 1.0 for DPNH) with increasing amounts of "sonic"

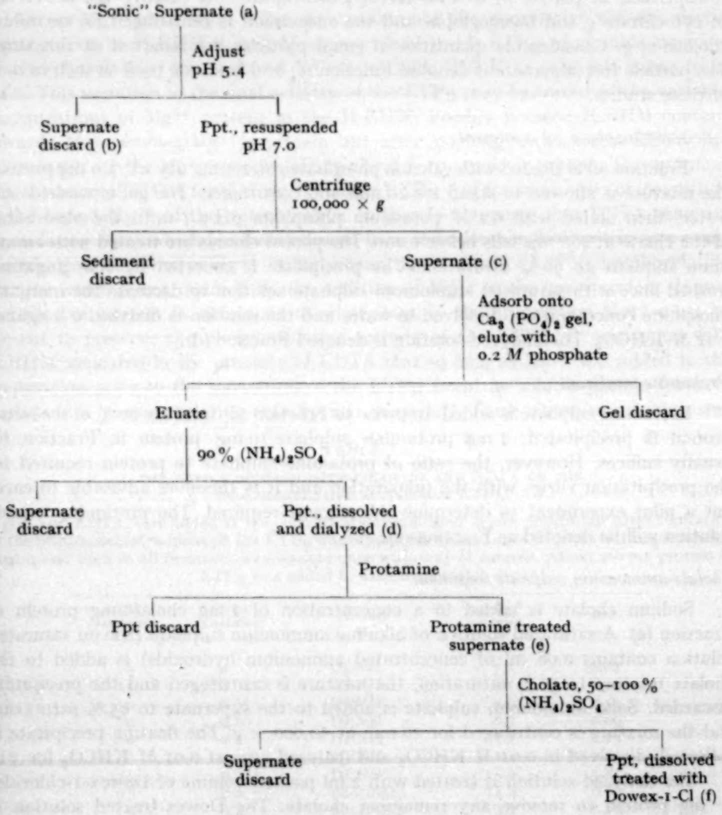
"Sonic" Supernate (a)
Adjust pH 5.4
Supernate discard (b)
Ppt., resuspended pH 7.0
Centrifuge 100,000 × g
Sediment discard
Supernate (c)
Adsorb onto $Ca_3(PO_4)_2$ gel, elute with 0.2 M phosphate
Eluate
Gel discard
90 % $(NH_4)_2SO_4$
Supernate discard
Ppt., dissolved and dialyzed (d)
Protamine
Ppt discard
Protamine treated supernate (e)
Cholate, 50–100 % $(NH_4)_2SO_4$
Supernate discard
Ppt, dissolved treated with Dowex-1-Cl (f)

Fig. 1. Fractionation of "Sonic" Supernate.

supernate, and then declines towards zero. Secondly the fractionation of the "sonic" supernate is accompanied by big losses in over-all activity. The phosphorylation factor becomes increasingly labile as purification proceeds and losses are sustained even during storage at low temperatures. On a protein basis the over-all purification to stage (f), Fig. 1, is 8 to 15 fold.

Acid precipitation

The "sonic" supernate is adjusted to pH 5.4 with 10% acetic acid and the precipitate formed is collected by centrifugation at 2000 × *g* for 10 min. The precipitate is resuspended in distilled water (one tenth the volume of the initial sonic supernate) and the pH of the suspension is adjusted to approximately 7.0. Then $KHCO_3$ is added to a final concentration of 0.01 *M*. The precipitation at pH 5.4 and resuspension at pH 7.0 in 0.01 *M* $KHCO_3$ are repeated to remove the last traces of cytochrome *c* and haemoglobin and the suspension is centrifuged for 90 min at 100,000 × *g*. Considerable quantities of small particles are removed at this stage. The particle free supernate is denoted Fraction (c) and has been used as such in most of these studies.

Calcium phosphate gel treatment

Fraction (c) is treated with calcium phosphate gel, 2.5 mg dry wt./1.0 mg protein; the mixture is allowed to stand for 20 min and centrifuged. The gel is washed with water, then eluted with 0.2 *M* potassium phosphate pH 7.3 until the absorbancy of the eluate at 280 mμ falls below 0.200. The pooled eluates are treated with ammonium sulphate to 90% saturation. The precipitate is collected by centrifugation, washed once with saturated ammonium sulphate solution to decrease the inorganic phosphate concentration, dissolved in water and the solution is dialyzed 6 h against 0.01 *M* $KHCO_3$. The dialyzed solution is denoted Fraction (d).

Protamine treatment

Protamine sulphate is added dropwise to Fraction (d) until 50–60% of the initial protein is precipitated; 1 mg protamine sulphate/10 mg protein in Fraction (d) usually suffices. However, the ratio of protamine sulphate to protein required for the precipitation varies with the preparation and it is therefore advisable to carry out a pilot experiment to determine the amount required. The protamine treated solution will be denoted as Fraction (e).

Cholate-ammonium sulphate treatment

Sodium cholate is added to a concentration of 1 mg cholate/mg protein of Fraction (e). A saturated solution of alkaline ammonium sulphate (100 ml saturated solution contains 0.08 ml of concentrated ammonium hydroxide) is added to the cholate mixture to 50% saturation, the mixture is centrifuged and the precipitate discarded. Solid ammonium sulphate is added to the supernate to 95% saturation and the mixture is centrifuged for 10 min at 50,000 × *g*. The floating precipitate is collected, dissolved in 0.01 *M* $KHCO_3$ and dialyzed against 0.01 *M* $KHCO_3$ for 3 h.

The dialyzed solution is treated with 2 ml packed volume of Dowex-1-chloride/10 mg protein to remove any remaining cholate. The Dowex-treated solution is denoted Fraction (f).

In the absence of cholate there is no clean separation of the activity by fractional precipitation with ammonium sulphate.

All high speed centrifugations were carried out in a Spinco Model L preparative ultracentrifuge. The centrifugal forces quoted are the values at the center of the centrifuge tubes. Protein solutions were maintained in the temperature range 0 to 5° during all manipulations except where otherwise indicated.

RESULTS

In Table I the phosphorylative activities of several different ETP_H preparations isolated in the presence of EDTA and Mg^{++} are compared. Particles prepared from H-BHM sonicated in the presence of EDTA are essentially devoid of phosphorylative activity. However, particles which have been sonicated in the presence of EDTA and excess Mg^{++} or of Mg^{++} alone show maximum phosphorylative activity. Sonic disintegration of H-BHM in 0.25 *M* sucrose alone yields ETP_H of variable capacity for phosphorylation; the observed P/O ratios with DPNH as substrate range from 0–1.6. This variation in the final activity of the ETP_H may be traced to the variable concentrations of Mg^{++} present in the H-BHM. Freshly isolated H-BHM contain upwards of 30 µmoles Mg^{++}/g protein but after washing with sucrose-EDTA (*cf.* EXPERIMENTAL) the Mg^{++} content falls to about 8–12 µmoles/g protein. The optimum concentration of EDTA for the inactivation of ETP_H is of the same order of magnitude as the bound Mg^{++} of the mitochondria. It therefore appears that EDTA may act by chelating Mg^{++} which has been released from the mitochondrial matrix as a result of sonication. Removal of Mg^{++} during preparation results in a loss of phosphorylative activity which is not restored by the addition of Mg^{++} to the assay system. It was therefore of interest to determine at what stage in the preparation Mg^{++} must be present to preserve the phosphorylative activity of the ETP_H. It was found with H-BHM sonicated in the presence of EDTA that so long as Mg^{++} was added to the preparation prior to the separation of the ETP_H from the soluble protein fraction, considerable phosphorylative activity was preserved. These observations suggested

TABLE I

THE EFFECT OF MAGNESIUM CHLORIDE AND EDTA ON THE PHOSPHORYLATIVE ACTIVITY OF ETP_H

$MgCl_2$ and EDTA were added at the concentrations indicated before sonication. After sonication of the mitochondrial suspension the ETP_H was separated, washed once with the initial suspending media and then in all instances was washed once with 0.25 *M* sucrose. About 2.0 mg protein of ETP_H was added to each manometer cup.

$MgCl_2$ (µmoles/ml)	EDTA (µmoles/ml)	Substrate	µmoles P esterified	P/O
0	0	DPNH	7.2	0.84
15	0	DPNH	16.1	1.77
0	0.05	DPNH	4.9	0.53
0	0.10	DPNH	0.7	0.09
0	0.2	DPNH	1.1	0.13
0	1.0	DPNH	1.2	0.11
15	0.2	DPNH	15.9	1.81
15	0.2	Succinate	14.4	0.87
0	0.2	Succinate	1.1	0.06

that a soluble component was being lost from ETP_H kept in the absence of Mg^{++}, and this indeed proved to be the case (*cf.* Table II).

TABLE II

RECONSTITUTION OF PHOSPHORYLATIVE ACTIVITY OF MODIFIED ETP_H BY SOLUBLE PROTEIN FRACTIONS

The fractions were derived as set out in Fig. 1. Each manometer cup contained 1.7 mg protein of modified ETP_H and the additions to individual manometer cups are as shown.

Additions (mg protein) Fraction	*Substrate*	*µmoles P esterified*	*P/O*
None	DPNH	2.1	0.14
(a), 0.42	DPNH	6.4	0.41
(a), 1.26	DPNH	9.1	0.58
(a), 3.78	DPNH	4.3	0.28
(b), 0.56	DPNH	5.6	0.35
(b), 1.12	DPNH	4.1	0.28
(b), 2.24	DPNH	1.7	0.11
(c), 0.26	DPNH	3.3	0.23
(c), 0.52	DPNH	10.5	0.77
(c), 1.56	DPNH	13.8	1.01
(c), 2.12–4.68	DPNH	—	0.94–1.05
None	Succinate	0.7	0.03
(a), 1.26	Succinate	7.9	0.32
(b), 0.56	Succinate	5.3	0.23
(b), 2.24	Succinate	0	0
(c), 0.52	Succinate	9.6	0.41
(c), 1.56	Succinate	11.2	0.49

ETP_H prepared in the presence of EDTA with no added Mg^{++} showed little capacity for coupled oxidation. However, the addition of the soluble protein fraction to the assay system restored much of this phosphorylation (*cf.* Table II). The presence of an inhibitor was also evident: high concentrations of Fraction (a) (*cf.* Fig. 1) inhibited phosphorylation. As shown in Table II, the activator and inhibitor are separated by acid fractionation of the sonic supernate at pH 5.4 (*cf.* Fig. 1). While Fraction (b) still displays some ability to reactivate the modified ETP_H, it clearly contains also a potent inhibitor of phosphorylation. On the other hand Fraction (c) is free from the inhibitor and with DPNH and succinate as substrates P/O ratios of 1.0 and 0.5 respectively are obtained in presence of added Fraction (c). In none of the reconstituted systems, in the presence of any of the soluble protein fractions so far obtained, have P/O ratios been observed exceeding 1.0 with DPNH as substrate, or 0.6 with succinate.

No consistent effect on the rates of oxidation has been observed with any of these soluble protein fractions. It would appear, therefore, that the factor functions directly in the phosphorylative mechanism.

Evidence for the recombination of the soluble component with modified ETP_H by means of Mg^{++} is presented in Table III. Modified ETP_H was suspended separately in 0.25 *M* sucrose; 0.25 *M* sucrose–0.01 *M* $MgCl_2$; 0.25 *M* sucrose–Fraction (c); 0.25 *M* sucrose–Fraction (c)–0.01 *M* $MgCl_2$; the particles were reisolated by centrifugation, washed with 0.25 *M* sucrose and then assayed. Only those particles treated with both Mg^{++} and Fraction (c) showed a significant increase in phosphorylative activity.

TABLE III

RECOMBINATION OF MODIFIED ETP_H WITH A SOLUBLE PROTEIN FRACTION THROUGH THE AGENCY OF Mg^{++}

*Fraction**	*Substrate*	*μmoles P esterified*	*P/O*
ETP_H	DPNH	2.6	0.18
$ETP_H + Mg^{++}$	DPNH	2.8	0.21
ETP_H + Fraction (c)	DPNH	3.6	0.27
$ETP_H + Mg^{++}$ + Fraction (c)	DPNH	11.0	0.86

* ETP_H (12 mg protein) was suspended in 7.0 ml of 0.25 *M* sucrose, the $MgCl_2$ to 15 μmoles/ml and Fraction (c) (32.4 mg protein) were added where indicated. The volumes were made to 12 ml; the mixtures centrifuged; then all sediments were well washed with 0.25 *M* sucrose; and finally resuspended in 0.25 *M* sucrose. 1.6 mg of ETP_H protein per manometer flask was then assayed.

Stabilization of modified ETP_H

Storage of the modified ETP_H in 0.25 *M* sucrose for 24 h either at 1° or $-10°$ usually results in a complete loss of the capacity for reactivation. This inactivation can be prevented by storage in the presence of a mixture of $MgCl_2$, GSH, ATP and succinate (*cf.* Table IV). There was a partial preservation of this activity in the presence of only some of these components but the medium which gave optimum results contains all four. Particles stored in this manner are still capable of some reactivation after about seven days.

TABLE IV

STABILIZATION OF MODIFIED ETP_H

The substrate was DPNH. A modified ETP_H preparation was divided into a number of samples and stored at 1° for 4 days in the presence of the additions mentioned in the table, except where indicated. 2.0 mg protein of ETP_H, were then assayed in the presence of 2.3 mg protein of Fraction (c). The concentrations of each of the components in the stored sample were ETP_H (10 mg/ml), $MgCl_2$ (5 μmoles/ml), GSH (2 μmoles/ml), ATP (2 μmoles/ml) and succinate (4 μmoles/ml).

Additions during storage	*μmoles P esterified*	*P/O*
None (assayed freshly prepared)	9.4	0.83
None	0	0
ATP–GSH	5.2	0.37
$MgCl_2$–GSH	5.8	0.43
$MgCl_2$–ATP	6.8	0.48
$MgCl_2$–GSH–ATP	9.2	0.64
$MgCl_2$–GSH–Succinate	7.8	0.54
$MgCl_2$–GSH–ATP–Succinate	10.3	0.71

Properties of the soluble component

The phosphorylation factor has been purified by conventional protein fractionation procedures as set out in Fig. 1. It is non-dialyzable, heat labile and is presumably protein in nature. Fraction (e) contains some lipide—of the order of 10 % of the total dry weight. Reliable assays of the lipide in Fraction (f) have not yet been obtained but significant amounts of lipide are known to be present.

Fraction (e) contains some RNA, of the order of $2 \cdot 10^{-3}$ μmole nucleic acid ribose/mg protein. However, the RNA is believed to be a contaminant, since in passing from Fraction (d) to Fraction (e) a 10 fold reduction in RNA concentration is observed while the factor is purified about 2 fold.

Fraction (c) shows some ATP'ase activity (about 0.070 µmole ATP/min/mg protein) but Fraction (e) which is approximately 4 times as active as Fraction c in coupling activity hydrolyzes only about 0.015 µmole ATP/min/mg protein. The ATP'ase activity of neither fraction is stimulated by 2,4-dinitrophenol.

It is difficult to follow quantitatively the purification of the factor as a strictly linear response of the modified ETP_H to the factor is not observed. Indeed it would seem that the protein is rather labile and quickly loses activity on storage. This loss is not a simple phenomenon, as the addition of aged fractions in increasing amounts to modified ETP_H seldom results in P/O ratios comparable to those obtained with freshly prepared fractions (*cf.* Table V). This phenomenon is not due to change in the particles since the same particles in the presence of fresh fractions show a higher P/O ratio than that observed in presence of aged fractions. Perhaps the protein factor undergoes a change on ageing such that it can still combine with the active sites of the modified ETP_H but is inactive with regard to phosphorylation, thus blocking the entry of active factor to the active sites.

TABLE V

EFFECT OF AGEING SOLUBLE FRACTIONS AND COMPARISON OF ACTIVITIES OF FRACTIONS OF DIFFERENT PURITY

The substrate was DPNH. Each manometer flask contained 1.8 mg protein of the modified ETP_H and the additions as indicated

Additions (mg protein)	*Period aged* (days)*	*µmoles P esterified*	*P/O*
None	0	1.7	0.09
None	4	1.0	0.06
Fraction (c), 0.4	0	4.6	0.26
Fraction (c), 0.8	0	9.1	0.49
Fraction (c), 1.6	0	11.6	0.69
Fraction (c), 3.0	0	11.8	0.65
Fraction (c), 0.8	4	4.7	0.32
Fraction (c), 1.6	4	7.5	0.49
Fraction (c), 3.0	4	7.9	0.53
Fraction (e), 0.15	0	6.1	0.38
Fraction (e), 0.3	0	10.7	0.65
Fraction (e), 0.6	0	13.8	0.87
Fraction (e), 1.2	0	15.1	0.91
Fraction (f), 0.07	0	5.8	0.37
Fraction (f), 0.21	0	11.9	0.71
Fraction (f), 0.56	0	14.5	0.88
Fraction (f), 1.12	0	16.0	0.98

* All fractions were assayed with the same batch of modified ETP_H. Fraction (c) and the enzyme were prepared and tested forthwith (day 0). Two days later Fraction (e) and 4 days later Fraction (f) and the above Fraction (c) were tested with ETP_H.

At this stage in the problem we can only make an approximate estimate of the degree of purification achieved by the procedures described. The relative activities of the fractions listed in Table V, indicate a 5–6 fold purification of Fraction (f) compared to the freshly prepared Fraction (c), and about 9–11 fold purification compared to Fraction (c) stored during the manipulations necessary to produce Fraction (f).

DISCUSSION

An important step in the preparation of ETP_H and modified ETP_H is the ageing and the freezing and thawing of the mitochondria before sonication. The function of this treatment is not clearly understood, but electron micrographs of these mitochondria and ETP_H suggest an explanation. The mitochondria are found to swell and lose some of their contents; the normally closely packed cristae are separated and some are fragmented while still within the mitochondrion. On sonication the outer membrane is ruptured and ETP_H is liberated into suspension. Morphologically ETP_H and modified ETP_H are double membrane fragments of cristae[9]. Other mitochondrial fragments with phosphorylative activity also possess this double membrane structure. Particles, enzymically more complex than ETP_H, which catalyze the oxidation of citric-acid cycle substrates with accompanying phosphorylations have been described as fragments of mitochondrial cristae enclosed within a limiting membrane[4].

By contrast ETP which is incapable of phosphorylation under any conditions consists of single membrane vesicles[4]. The double membrane structure, which is found in all those sub-mitochondrial fragments so far prepared which are capable of oxidative phosphorylation, appears to be a *sine qua non* for phosphorylative activity and it may be a token of the preservation of some spatial arrangement essential to this process.

A number of sub-mitochondrial preparations with phosphorylative activity have been described from various laboratories[2, 10–13]. However, although their properties are somewhat different, all are particulate in nature. The insoluble character of the enzyme complex has made progress in the problem difficult. The resolution of ETP_H into a three component system, viz. modified particles capable of aerobic oxidation, a soluble protein fraction and Mg^{++}, opens the way to a more conventional approach to the problem. Recent reports on microbial systems suggest that this difficulty is also being resolved in that field[9].

The purified protein fraction does not function in the oxidative process but appears to be directly involved in phosphorylation. As yet we are unable to assign any more precise role to the factor, but the presence of appreciable concentrations of lipide associated with the activity suggests the possible involvement of a lipoprotein.

A requirement by mitochondria for Mg^{++} in oxidative phosphorylation has long been recognized and particles derived from liver mitochondria have been described with an absolute dependency on Mg^{++} for phosphorylation[12]. Another function or a more precise role can now be assigned to magnesium: evidently it binds the soluble protein factor to the electron transport system.

PULLMAN *et al.*[14] and PENEFSKY *et al.*[15] have recently described the preparation from beef-heart mitochondria of a particle not unlike modified ETP_H which also shows a requirement for a soluble factor to achieve coupled oxidation of succinate. This particle is obtained by disintegration of mitochondria in a Nossal shaker. The soluble factor is active only in presence of Mg^{++}. The coupling activity of the factor runs parallel with ATP'ase activity which is stimulated by 2,4-dinitrophenol. The relation of the Pullman-Penefsky factor to the factor described in this communication is still not clear.

ACKNOWLEDGEMENTS

We wish to express our gratitude to Dr. D. E. Green for counsel and encouragement throughout this work. We are also indebted to Dr. R. L. Lester for the ATP'ase determinations and to Miss B. Wagner for valuable technical assistance.

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SYNTHESIS OF MACROMOLECULES IN SYNCHRONOUSLY DIVIDING BACTERIA

FRED E. ABBO* AND ARTHUR B. PARDEE

Biochemistry Department and Virus Laboratory, University of California, Berkeley, Calif. (*U.S.A.*)

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SUMMARY

1. A population of synchronously dividing *Eschericha coli B* was obtained by a filtration method which does not appear to alter the metabolism of the bacteria.

2. In such a synchronous bacterial population it was found that the synthesis of deoxyribonucleic acid, ribonucleic acid, protein, and induced β-galactosidase proceeded exponentially and continuously throughout the entire division cycle.

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* Present address: McArdle Memorial Laboratory, University of Wisconsin, Madison 6, Wisconsin.

INTRODUCTION

Previous workers, using exponentially growing bacterial cultures synchronized by means of temperature fluctuations[1,2], thymine[3] or deoxyribose deprivation[4], or filtration[5], have uniformly reported a non-continuous, or step-wise, synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or protein through the division cycle. However, the conditions used have been suspected of introducing spurious metabolic effects. YOUNG AND FITZ-JAMES[6], on the other hand, have reported a continuous synthesis of DNA in a bacterial culture synchronized by germination of spores. Since the bacteria in this case were not in the exponential stage of growth, the question of synthesis in exponentially growing cultures remains unsettled. It is therefore of interest to study an exponentially growing, synchronously dividing bacterial population whose cell metabolism has been unaltered. The filtration method of obtaining a synchronously dividing population[5], as used in this study, appears to fulfill this requirement; and this method was therefore applied to measurements of synthesis of macromolecules by the bacteria.

In a discussion of synchronously dividing populations, it is important to distinguish those bacteria undergoing unbalanced growth from those undergoing balanced growth. The former, exemplified by temperature or nutrient-deprivation induced synchrony, we designate generically as *forced synchrony*, and specifically we shall refer to such a population with the transitive adjective *synchronized*. The latter, exemplified by a synchronously dividing population obtained by the filtration technique, we designate generically as *unforced synchrony* and refer to such a population with the intransitive adjective *synchronous*. We use the term "balanced growth" as defined by CAMPBELL[7], viz., the doubling of every extensive property of the cell within the time-period of one division cycle.

METHODS

Bacterial strain and medium

E. coli, strain B was used in the experiments reported here. The medium, a modification of the Hershey-Chase glycerol–lactate medium[8], contained, per liter, 5 ml glycerol, 5 g NaCl, 2 g KCl, 1 g NH_4Cl, 203 mg $MgCl_2$, 1.1 mg $CaCl_2$, 10 mg gelatin, 71.3 mg $K_2HPO_4 \cdot 3H_2O$, 76.6 mg $MgSO_4 \cdot 7H_2O$, and 0.1 M tris(hydroxymethyl)aminomethane at pH 7.0–7.2.

Synchronization technique

The synchronization technique was essentially that of MARUYAMA AND YANAGITA[5], with slight but important modifications. The principle of the technique is the filtration of a bacterial suspension through 24 layers of filter paper and the collection of the bacteria which initially pass through into the filtrate. This technique selects those small cells which have just completed division, and therefore provides a synchronously dividing population. The entire procedure was conducted in a room at 37° so that the bacteria were never exposed to temperature variations which could alter the relative rates of synthesis of their components. The bacteria were grown overnight, from an agar slant, in the above medium with shaking at 37°, to a concentration of 1 to $2 \cdot 10^8$ bacteria/ml. 10 ml of this suspension were then transferred into 200 ml of fresh medium, the bacteria were grown to a concentration of 1 to $2 \cdot 10^8$/ml, and then directly filtered to select the smallest bacteria. The filtration apparatus consisted of a Buchner funnel, suction flask, filter paper pile, and a leucite ring clamped down on the paper pile to prevent seepage of bacteria down the sides of the filter paper pile. The filter paper pile, from bottom to top, consisted of the following Whatman filter papers: one No. 42, twenty No. 1, one No. 42, two No. 1. The culture was poured directly onto the filter paper pile and filtered under suction till a turbid filtrate was observed, using additional fresh medium to elute the bacteria. The bacteria in the filtrate were then recovered by filtration onto a millipore filter and resuspended in fresh medium. Typical times involved: filtration to turbidness, 10 to 20 min; collection and resuspension of cells in fresh medium, 4 to 8 min. Total time required: 14 to 28 min. It is noteworthy that the cells were well oxygenated at all times, since fresh oxygenated medium was constantly percolating through the filter paper pile.

The cell bacterial counts were made with the Petroff-Hausser counting chamber. The light scattering experiment was performed using the Aminco-Bowman spectrophotofluorometer. The method is a modification of that described by STARKA AND KOZA[9]. In this case, samples were prepared by appropriately diluting an aliquot of the growing bacteria to a final concentration of approximately $6 \cdot 10^6$ bacteria/ml in phosphate buffer, pH 6.3, containing 2 % formaldehyde. The % transmission at a 90° angle was measured, using a 0.03 scale and light of wavelength 450 mμ.

Biochemical determinations

Protein, RNA, and DNA were determined colorimetrically on samples which had been precipitated with 10 % trichloracetic acid and washed with cold 5 % trichloracetic acid. The FOLIN[10], orcinol[11], and BURTON-DISCHE[12] methods were used, respectively.

[^{3}H]thymidine (obtained from Schwartz Laboratories, 1900 μC/μmole) or [2-^{14}C]leucine (7.95 μC/μmole) incorporation was determined by precipitating the bacteria in cold 10 % trichloracetic acid, allowing the precipitate to stand at 2° for at least 30 min, then filtering on a millipore filter, washing, drying, and counting in windowless-flow or end-window Geiger-Muller counters, respectively. It was determined that the sample sizes used were such as to provide reproducibly thin counting conditions. Also, the uptake of radioactivity in each case was linear with time and cell concentration over a 10-min period in non-synchronous cultures.

The synthesis of β-galactosidase was induced by incubating the growing bacteria in growth medium containing $5 \cdot 10^{-4}$ *M* isopropyl-thio-β-D-galactoside for 6.5 min at 37°. Enzyme synthesis was stopped by adding one drop of toluene to 1 ml of culture, mixing, and agitating at 37° for 30 min. The β-galactosidase activity of the extract was determined by the color formed on hydrolysis of *o*-nitrophenyl-β-D-galactoside[13].

RESULTS

Fig. 1 presents the results of two independent methods of measuring the synchrony obtained by the filtration technique. A qualitative examination of the plotted points in this and the other figures of this paper clearly shows the synchrony. All of the bacteria appear to divide within 25 % of the division cycle. A statistical analysis was made of the data in Fig. 1 as follows: The two alternative hypotheses were considered:

on the one hand, that the numbers of cells increased in a step-wise fashion as indicated by the depicted step-functions; on the other hand, that the numbers of cells increased exponentially, represented by a straight line, drawn qualitatively to provide the best fit for the experimental points through the two cycles shown. For each set of points, the variance from each hypothesis was calculated, the ratio of these variances obtained, and the "F" test applied[14]. It was found that the data from both the Petroff-Hausser counts and the light scattering measurements conformed to the step-function at the 99 % confidence level. Furthermore, on thirty replicate Petroff-Hausser counts, the relative standard deviation was found to be 6 %; and therefore the relative standard deviation from the step-function, 5 %, is accounted for by the variability of the measurements.

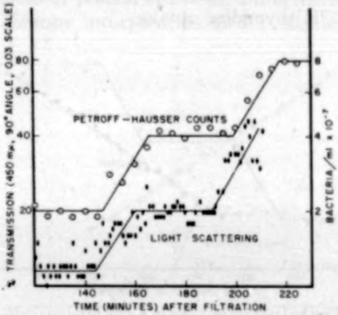

Fig. 1. Measurement of synchronous growth by Petroff-Hausser counts and light scattering.

The division cycles shown represent third and fourth generations. This duration of synchrony exceeds the limit predicted by CAMPBELL[7] on the basis of the standard deviation of the generation times of individual bacteria as determined by POWELL[15]. However, POWELL also showed that the standard deviation of the generation time varies with bacterial strain, medium, and family clone, being as low as 0.07 of a division cycle in one case of *B. coli anaerogenes*, in contrast with the 0.3 figure used by CAMPBELL in his calculations. We estimate, on the basis of the duration of our synchrony and CAMPBELL's calculated theoretical curves, that the standard deviation of the generation time in our cultures was less than 0.1 of a division cycle.

Fig. 2 shows the results of bacterial counts and of a colorimetric determination of DNA in a synchronous culture from which 10-ml aliquots were taken every 5 min. This is typical of results of several such determinations. DNA appears to increase throughout the growth cycle.

The linear curve obtained on a semi-logarithmic plot implies an exponential increase of DNA throughout the cycle. From such a plot, or from a plot of the data on ordinary linear graph paper, one cannot distinguish easily between a series of linear increases and an exponential curve, since the two curves differ by about 6 % at most. Resort was therefore made to "differential" measurements to measure the rates of synthesis. The rate of DNA synthesis must increase exponentially if the total DNA increases exponentially throughout the division cycle, since the derivative of an exponential is an exponential. On the other hand, if the total DNA increases linearly, the rate must remain constant throughout one growth cycle, and then double during the following cycle. Furthermore, if total DNA does not increase at any time, the rate must fall to zero. Therefore, DNA synthesis was measured over a short time interval, by [^{3}H]thymidine uptake.

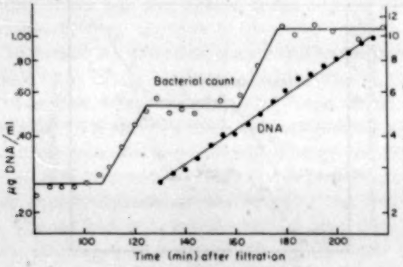

Fig. 2. Total DNA (Burton-Dische) per ml *vs.* time in a synchronous culture. The time of completion of filtration is taken as zero.

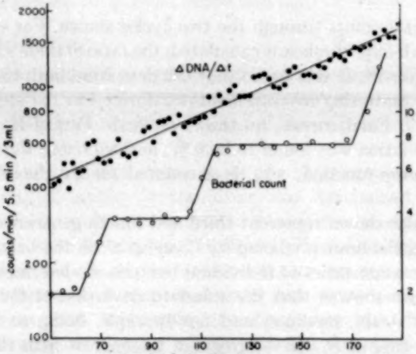

Fig. 3. Differential rate of DNA synthesis ([^{3}H]thymidine uptake) *vs.* time in a synchronized culture.

The differential rate of DNA synthesis was determined directly by withdrawing 3-ml aliquots from 270 ml of a growing, synchronous culture every 2 min, incubating each aliquot for 5.5 min at 37° with shaking, with 0.5 ml of medium containing 3.5 µC of [^{3}H]thymidine and 3.5 µg unlabeled thymidine/ml. At the end of this short time interval the incorporation of [^{3}H]thymidine into DNA was stopped by adding 2 ml of 20 % TCA (trichloracetic acid) and placing the mixture in an ice bath for at least 30 min. The sample was then prepared and counted as described under METHODS, and the amount of uptake of [^{3}H]thymidine was taken as a measure of the rate of DNA synthesis at the time the aliquot was withdrawn from the master culture. A plot of the results is shown in Fig. 3. It is seen that the rate of synthesis of DNA increases exponentially, hence likewise the total DNA.

RNA and protein were determined colorimetrically on aliquots taken throughout the division cycle, the results being depicted in Figs. 4 and 5. The synthesis of these macromolecules is seen likewise to be continuous and apparently exponential. Furthermore, to confirm the pattern of protein synthesis, a differential rate experiment was performed utilizing [^{14}C]leucine incorporation into TCA-precipitable material as a measure of protein synthesis. 2 ml of synchronous culture were withdrawn every 2 min and incubated 4.5 min in growth medium containing final [^{14}C]leucine corresponding to 9000 counts/min/ml and 0.4 µg/ml unlabeled leucine and precipitated and counted as previously described. The result, shown in Fig. 6, confirms the colorimetric experiment. It might be objected that TCA-precipitable [^{14}C]leucine is in the form of leucine bound to soluble RNA[16], and therefore does not represent protein incorporation. However, assuming 20 % of the cell RNA to be in the form of soluble RNA and 5 % of these molecules to be involved in the binding of the specific amino

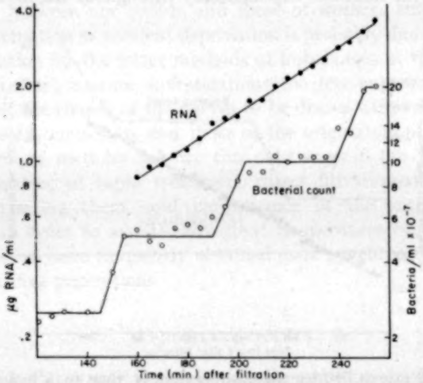

Fig. 4. Total RNA (orcinol) per ml *vs.* time in a synchronous culture.

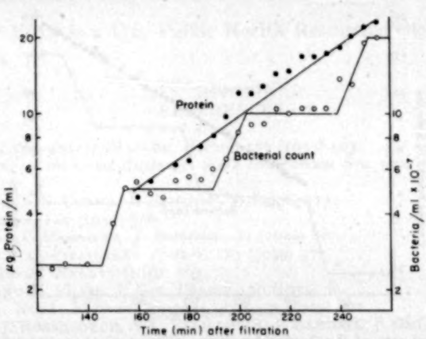

Fig. 5. Total protein (Folin) per ml *vs.* time in a synchronous culture.

acid leucine, a calculation shows that under the conditions of the experiment this contribution to the measured radioactivity would be negligible.

The rate of formation of the inducible enzyme β-galactosidase was determined by withdrawing one ml of synchronous culture every 2 min and proceeding as described under METHODS. The result, shown in Fig. 7, shows that the ability to form this inducible enzyme increases exponentially throughout the division cycle.

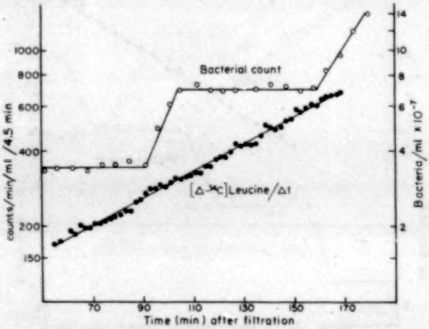

Fig. 6. Differential rate of [^{14}C]leucine incorporation *vs.* time in a synchronous culture.

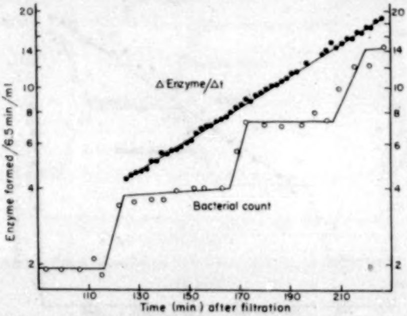

Fig. 7. Differential rate of β-galactosidase formation *vs.* time in a synchronous culture.

DISCUSSION

We have shown that the synthesis of DNA, RNA, protein and inducible β-galactosidase proceeds exponentially and continuously throughout the division cycle of a synchronous population of *E. coli*. We furthermore consider that this represents the time-course of synthesis of these macromolecules within the individual bacterium. The alternative explanation would be that the macromolecules are synthesized during only a fraction of the division cycle, and that this interval of synthesis (and its rate) varies from bacterium to bacterium in such a way that the summated effect in the population produces an apparent continuous synthesis. However, in the case of DNA at least, this unlikely alternative explanation is demonstrably untenable, in view of the recent work of Schaecter *et al.*[17] and McFall and Stent[18]. These workers utilized brief exposures to ^{32}P and [^{3}H]thymidine, respectively, in non-synchronous bacterial cultures to demonstrate a continuous synthesis of DNA in the individual bacterium.

The disparity between our results and those of workers employing synchrony by temperature fluctuation or nutrient deprivation is probably due to the introduction during synchronization by the latter methods of imbalances in the ratios of macromolecules. Furthermore, in some investigations too few experimental points were presented to permit the shapes of the curves to be drawn with certainty. To explain the disparity between our results and those of the originators of the filtration synchronization technique used by us[5], we can only suggest the importance of our modifications consisting of rapid treatment, direct filtration of the cells without previously concentrating them, and performance of the entire synchronization procedure at 37° in order to avoid inadvertent temperature cycling of the cells. It is noteworthy that we have frequently obtained good synchrony which has endured for at least four or five generations.

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ACTION DE LA 8-AZAGUANINE SUR LA SYNTHÈSE DES PROTÉINES ET DES ACIDES NUCLÉIQUES CHEZ *BACILLUS CEREUS*

H. CHANTRENNE ET S. DEVREUX

Laboratoire de Chimie biologique, Faculté des Sciences, Université de Bruxelles (Belgique)

(Reçu le 26 Juin, 1959)

SUMMARY

Effect of 8-azaguanine on the synthesis of proteins and nucleic acids in B. cereus

In the presence of 8-azaguanine, protein synthesis is strongly inhibited and the formation of constitutive penicillinase is completely abolished.

Low concentrations of azaguanine which are sufficient to block penicillinase formation do not change the rate of DNA synthesis. Higher concentrations cause an initial slight inhibition which becomes much stronger after about one doubling of the DNA.

Azaguanine increases the rate of RNA formation while being incorporated into RNA. At low concentration the analogue is incorporated for a limited time only; later a large part of it is rejected from the RNA.

The RNA of bacteria which have incorporated azaguanine is more labile in acid medium than normal RNA.

The effects of azaguanine on *B. cereus* resemble those of chloramphenicol on *E. coli* and *S. aureus*.

INTRODUCTION

Les travaux de LASNITSKI, MATTHEWS ET SMITH[1-3] et ceux de MANDEL[4] et de MARKHAM[5] ont établi que l'azaguanine s'incorpore dans les acides ribonucléiques de *Bacillus cereus* à la place de la guanine et qu'elle inhibe la croissance de ces bactéries, telle qu'on peut l'évaluer en mesurant la densité optique des suspensions bactériennes.

Nous avons souligné ailleurs[6-8] que l'inhibition partielle de la "croissance" que l'on mesure ainsi masque des perturbations profondes de la synthèse des protéines et des acides nucléiques. Nos résultats expérimentaux n'ont été présentés jusqu'ici que d'une façon sommaire; on en trouvera les détails dans le présent article, en même temps que quelques observations nouvelles et une discussion plus complète de l'ensemble de nos résultats.

MATÉRIEL ET MÉTHODES

Bactérie: Mutant de *Bacillus cereus* produisant la pénicillinase comme enzyme constitutif. Ce mutant fut isolé d'une souche de *B. cereus* N.C.T.C. 569 selon la méthode de KOGUT *et al.*[9].

Milieu de culture: Milieu contenant un hydrolysat de caséine et préparé selon POLLOCK *et al.*[10].

Milieu d'expérience: Le milieu de culture additionné de citrate et de Tween 80, selon TORRIANI[11]. Incubation dans des fioles de 100 ml contenant 20 ml de suspension bactérienne, et agitées en thermostat à 30°.

Dosage des protéines: Selon LOWRY *et al.*[12]. Etalonnage avec une solution de tyrosine. Les résultats sont exprimés en quantité de tyrosine donnant la même coloration que l'échantillon dosé.

Dosage des acides ribonucléiques: Méthode à l'orcinol, version de MILLER *et al.*[13]. Etalonnage avec une solution d'ARN de levure dont la concentration est déterminée par mesure de l'extinction à 260 mμ en admettent qu'une extinction de 1.00 correspond à 34.6 μg d'ARN/ml.

Dosage des acides désoxyribonucléiques: Méthode de *Ceriotti*[14]. Etalonnage avec de l'ADN de thymus de veau.

Dosage de la pénicillinase: Selon TORRIANI[11].

Incorporation de substances marquées dans les protéines et les acides nucléiques: Sauf indications contraires, les précurseurs marqués utilisés dans le présent travail étaient suffisamment spécifiques des substances dont on étudiait la synthèse pour qu'il suffît de déterminer la quantité d'isotope radioactif incorporé dans les substances précipitées par l'acide trichloracétique.

Des échantillons de la suspension bactérienne étaient mélangés à un volume double d'une solution glacée d'acide trichloracétique dans laquelle on avait dissous au préalable un peu d'une substance non marquée identique, à cela près, au précurseur radioactif utilisé. Le mélange était conservé 30 min dans la glace fondante. Le précipité était ensuite transféré quantitativement sur un filtre "Millipore" de 25 mm, lavé deux fois sur le filtre avec la même solution d'acide trichloracétique, une fois encore avec une solution à 5 % de ce même acide, après quoi le filtre était montré sur un support adéquat et séché à l'air. La radioactivité du précipité était mesurée à l'aide d'un compteur à fenêtre mince.

Substances marquées: DL-[^{35}S]méthionine, DL-[1-^{14}C]valine, DL-[1-^{14}C]leucine, Acide DL[1-^{14}C]glutamique, acide L-aspartique et L-phénylalanine uniformément marqués, [2-^{14}C]uracile, [8-^{14}C]guanine ont été fournis par le Radiochemical Centre, Amersham, Grande Bretagne.

La 8-[2-^{14}C]azaguanine nous a été aimablement donnée par le Dr. G. B. BROWN; elle avait été préparée par le Southern Research Institute pour l'Atomic Energy Commission.

Autres produits: 8-azaguanine: Light's et California Foundation. Guanosine: Schwarz.

RÉSULTATS EXÉPRIMENTAUX

Inhibition de la synthèse des protéines par l'azaguanine

La présence d'acides aminés et de sucres aminés dans les parois cellulaires de *B. cereus* complique l'étude de la synthèse des protéines chez cette bactérie, car elle interdit l'emploi de plusieurs méthodes classiques de dosage (par exemple la méthode à la ninhydrine) et restreint considérablement le choix des acides aminés marqués utilisables comme précurseurs spécifiques des protéines.

Les parois cellulaires de *B. cereus* contiennent, à côté de trois hexosamines, quatre acides aminés: alanine, acide diaminopimélique, acide glutamique[15] et acide aspartique[16]; il est évident que ces acides aminés et ceux qui peuvent leur donner naissance doivent être exclus dans les expériences d'incorporation dans lesquelles les mesures portent sur la radioactivité du précipité trichloracétique, et que de très grandes précautions doivent être prises pour éviter toute confusion entre synthèse des protéines et formation des parois cellulaires[17, 18].

La Fig. 1 montre l'effet de 36 µg d'azaguanine/ml sur la synthèse des protéines dans une suspension de *B. cereus*. Le temps 0 correspond au moment de l'addition de l'azaguanine à une suspension de bactéries en croissance exponentielle. Les protéines ont été dosées par la méthode de LOWRY sur l'extrait alcalin du précipité trichloracétique.

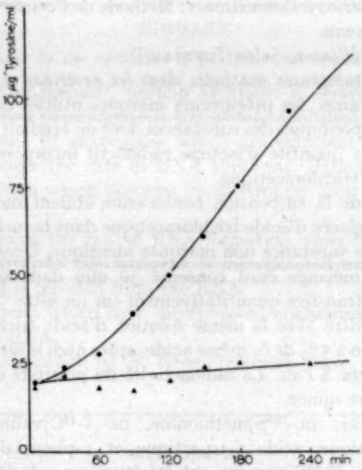

Fig. 1. Inhibition de la synthèse des protéines par l'azaguanine. Les protéines ont été dosées par la méthode de LOWRY *et al.*[12]. ● : pas d'azaguanine. ▲ : 36 µg d'azaguanine/ml au temps 0.

La Fig. 2a représente les résultats d'une expérience dans laquelle 36 µg d'azaguanine ont été ajoutés à l'instant indiqué par la flèche à des suspensions de bactéries auxquelles on avait ajouté respectivement de la DL-[1-^{14}C]valine et de la DL-[1-^{14}C]leucine (0.06 µmoles et 0.3 µC/ml). Le milieu contenait de l'hydrolysat de caséine à raison de 20 mg/ml. On trouvera dans la Fig. 2b et dans d'autres publications de ce laboratoire[6,8] les résultats d'expériences semblables dans lesquelles de la L-phenylalanine uniformément marquée ou de la DL-[^{35}S]méthionine ont été utilisées.

Dans toutes ces expériences, les résultats sont qualitativement les mêmes: l'azaguanine à 36 µg/ml inhibe aussi bien la synthèse des protéines telle qu'on la mesure selon la méthode de LOWRY[12] que l'incorporation de méthionine, valine, phénylalanine ou leucine. MANDEL[19] a également observé une inhibition de l'incorporation de ^{35}S donné sous forme de sulfate.

Des quantités 10 fois plus faibles d'azaguanine (3.6 μg/ml) produisent aussi une inhibition très nette de la synthèse des protéines (Fig. 3). Les résultats d'une expérience dans laquelle les protéines ont été dosées selon LOWRY ont été publiés ailleurs[7]. Dans certaines expériences dans lesquelles des concentrations voisines de 3 μg/ml ont été utilisées, on a observé une reprise de la synthèse des protéines après une période d'inhibition plus ou moins longue; nous y reviendrons dans une publication ultérieure consacrée à la restauration de la synthèse des protéines après inhibition par l'azaguanine.

La Fig. 3 montre l'effet de faibles concentrations d'azaguanine à la fois sur l'incorporation de L-phénylalanine marquée dans les protéines et sur la synthèse d'une protéine définie, la pénicillinase. Dans la souche utilisée, la pénicillinase est un enzyme constitutif, une protéine parmi tant d'autres que la bactérie produit normalement. Il est visible que la production de cet enzyme est complètement supprimée alors que l'incorporation de L-phénylalanine n'est que partiellement inhibée par les faibles concentrations d'azaguanine utilisées. On peut faire la même constatation en comparant l'action de l'azaguanine, même à forte concentration, sur la synthèse de l'enzyme et sur l'incorporation de méthionine dans les protéines[6].

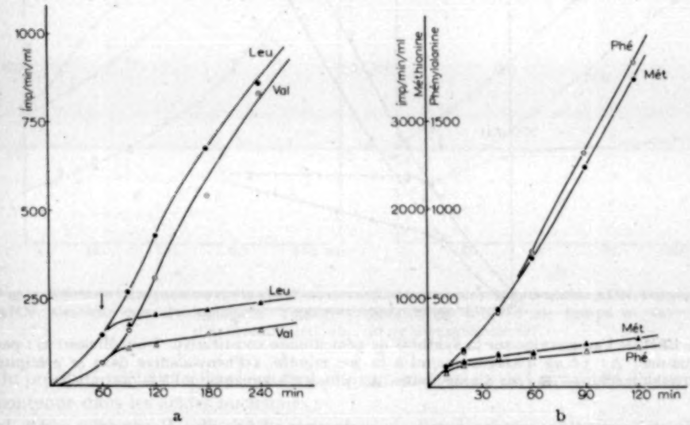

Fig. 2. Inhibition de l'incorporation d'acides aminés marqués dans le précipité trichloracétique. (a) Points noirs: DL-[1-^{14}C]leucine. Points clairs: DL-[1-^{14}C]valine. Cercles: pas d'azaguanine. Triangles: azaguanine (36 μg/ml) ajoutée à l'instant indiqué par la flèche. (b) Points noirs: DL-[^{35}S]méthionine. Points clairs: L-phénylalanine uniformément marquée. Cercles: pas d'azaguanine. Triangles: azaguanine (36 μg/ml) ajoutée au temps 0.

Effets de l'azaguanine sur la synthèse des acides nucléiques

L'addition de 3.6 μg d'azaguanine/ml à une suspension de *B. cereus* en croissance exponentielle qui contenait au moment de l'addition 0.3 mg de bactéries (poids sec)/ml, stimule la synthèse des acides ribonucléiques, mais n'exerce pas d'action mesurable sur celle des acides désoxyribonucléiques. Par exemple, dans l'expérience reprise dans la Fig. 4a, la synthèse de l'ADN s'est poursuivie à la vitesse normale en présence d'azaguanine. La vitesse de synthèse de l'ARN était à peu près doublée pendant les 100 premières minutes d'action de l'analogue, pour revenir ensuite à une valeur normale. On trouvera les résultats d'une autre expérience de ce type dans une autre publication[7].

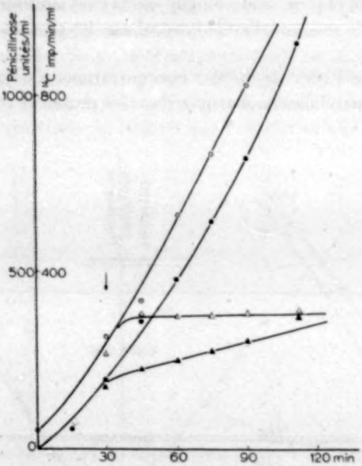

Fig. 3. Effet de l'azaguanine sur la synthèse de pénicillinase constitutive. Pénicillinase: ○ : pas d'azaguanine. △ : 3.6 μg d'azaguanine/ml à la 30e minute. L-Phénylalanine dans le précipité trichloracétique: ● : pas d'azaguanine. ▲ : 3.6 μg d'azaguanine/ml à la 30e minute.

En présence d'azaguanine à une concentration 10 fois plus élevée (36 μg/ml), la synthèse d'ADN peut se poursuivre à la vitesse normale pendant un certain temps, ou subir dès le début une légère inhibition. Dans tous les cas, on observe un ralentissement très prononcé de la synthèse d'ADN vers le moment où la quantité d'ADN a doublé mais la synthèse se poursuit à faible vitesse pendant plusieurs heures (Fig. 4b).

La synthèse d'ARN ne subit aucune inhibition en présence d'azaguanine à concentration élevée (36 μg/ml); il arrive même que la quantité d'ARN formé soit plus élevée que dans le témoin[8]. Il faut souligner ici que nos résultats expriment la quantité d'ARN par ml de suspension, et que les bactéries-témoins sont en pleine croissance tandis que les bactéries soumises à l'action de fortes concentrations d'azaguanine cessent bientôt de se multiplier et qu'elles ne font plus de protéines. Il en résulte que, par bactérie, la production d'ARN est toujours beaucoup plus élevée en présence d'azaguanine qu'en son absence.

Pour une même quantité d'ADN, les bactéries traitées par l'azaguanine contiennent donc beaucoup plus d'ARN et beaucoup moins de protéines que les bactéries normales[8].

Incorporation d'azaguanine dans l'ARN

A des suspensions de bactéries en croissance exponentielle, ajoutons de la [2-^{14}C] azaguanine (4 μC/mg) à diverses concentrations et déterminons comment évolue, au cours du temps, la quantité totale de ^{14}C contenu dans les acides nucléiques d'un ml de suspension. L'azaguanine s'incorpore telle quelle dans l'ARN. Elle ne subit aucune dilution isotopique, puisque la bactérie ne produit pas d'azaguanine. La radioactivité du précipité trichloracétique est donc une mesure correcte de la quantité d'azaguanine contenue dans les acides nucléiques.

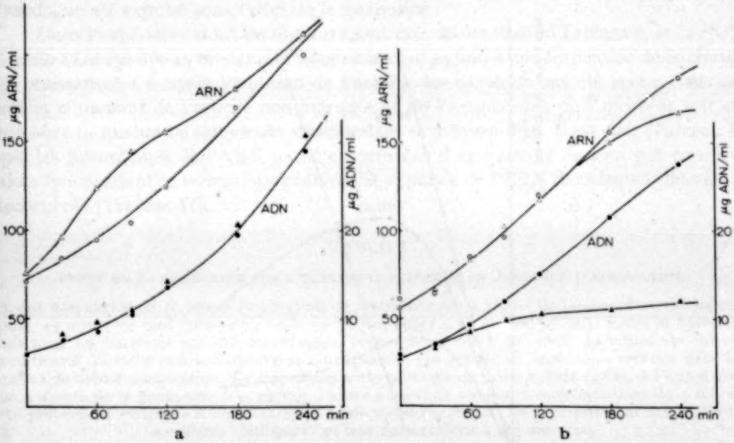

Fig. 4. Effets de l'azaguanine sur la synthèse des acides nucléiques. Points noirs: ADN. Points clairs: ARN. Cercles: pas d'azaguanine. Triangles: azaguanine ajoutée au temps o. (a): 3.6 μg d'azaguanine/ml. (b): 36 μg d'azaguanine/ml.

En présence de 36 μg d'azaguanine/ml, l'incorporation se poursuit presque linéairement pendant plusieurs heures (Fig. 5) ainsi que MANDEL[4] l'avait déjà observé. Mais lorsque la concentration de l'analogue est 10 ou 20 fois plus faible, l'incorporation ralentit bientôt, et la quantité totale d'azaguanine contenue dans l'acide nucléique passe par un maximum pour décroître régulièrement ensuite.

Cette expérience montre que si les concentrations d'azaguanine ne sont pas trop élevées, les bactéries peuvent éliminer la purine étrangère après qu'elle se soit incorporée pendant quelque temps dans leur ARN.

MATTHEWS ET SMITH[2,3] et MANDEL ET MARKHAM[4,5] ont montré que l'addition d'une purine ou d'un nucléoside purique normal à une suspension de *B. cereus* intoxiqué par l'azaguanine provoque l'expulsion de l'analogue qui s'était incorporé dans les ARN. Il est donc vraisemblable que les observations rassemblées dans la Fig. 5 relèvent du même phénomène, et que lorsque la concentration de l'azaguanine n'est pas trop élevée, les purines produites par la bactérie elle-même déplacent l'analogue et provoquent son élimination, comme le feraient des purines exogènes.

Si nous rapprochons cette observation de celles que nous avons rapportées plus haut concernant l'action de faibles concentrations d'azaguanine sur la synthèse des acides nucléiques, nous constaterons que tout se passe comme si de l'ARN supplémentaire s'accumulait dans les bactéries pendant que de l'azaguanine s'y incorpore, et comme s'il cessait de s'accumuler lorsque l'azaguanine est rejetée. L'action de faibles concentrations d'azaguanine sur la synthèse d'ARN est semblable à celle de concentrations plus fortes, mais elle est de plus courte durée parce que les bactéries se débarrassent de l'azaguanine après un certain temps.

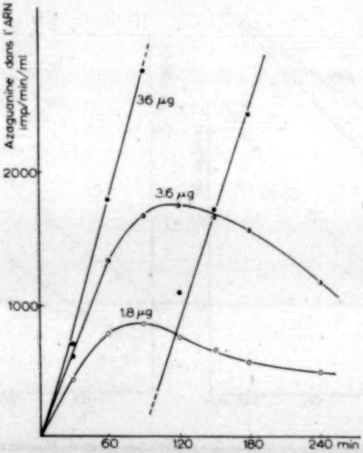

Fig. 5. Evolution de la quantité totale de[2-^{14}C]azaguanine contenue dans les acides nucléiques d'un ml de suspension bactérienne, pour les trois concentrations d'azaguanine indiquées. 1000 coups/min correspondent à 1.25 μg d'azaguanine.

Propriétés métaboliques de l'ARN formé en présence d'azaguanine

L'azaguanine inhibe donc fortement la synthèse des protéines chez *B. cereus* et elle provoque l'accumulation d'ARN dans la bactérie. Cet ARN excédentaire est anormal, puisqu'une partie de la guanine y est remplacée par de l'azaguanine[5]. La purine anormale n'exerce que tardivement une action sur la synthèse de l'ADN, et elle n'a pas d'effet sur la formation des constituants des parois cellulaires[8].

Toutes ces observations rappellent l'action de la chloromycétine sur *S. aureus*: l'antibiotique inhibe en effet la synthèse des protéines, sans affecter celle des parois cellulaires[17, 18] et il provoque l'accumulation d'un ARN anormal. L'ARN qui s'accumule chez *E. coli* en présence de chloromycétine se distingue de l'ARN normal par son instabilité métabolique; lorsque les bactéries sont transférées dans un milieu débarrassé de chloromycétine l'ARN qui s'était accumulé en présence de l'antibiotique est détruit et ses produits de dégradation sont rejetés dans le milieu[23, 27].

Dans le but de comparer l'action de l'azaguanine à celle de la chloromycétine, nous avons cherché à savoir ce qu'il advient de l'ARN formé en présence d'azaguanine lorsque l'effet de la purine anormale est supprimé par addition de guanosine. On pourrait penser que l'azaguanine est rejetée[2–5] parce que les ARN anormaux sont détruits.

Si les ARN des bactéries en croissance ne présentent pas de renouvellement notable, il est facile de distinguer les ARN qui existaient avant l'addition de l'azaguanine de ceux qui se sont formés en sa présence; il suffit d'ajouter au milieu de l'uracile marquée pendant une période bien choisie. Nous avons étudié par ce moyen le sort des pyrimidines des ARN formés en présence d'azaguanine lorsque l'analogue est expulsé sous l'effet de la guanosine.

Dans l'expérience dont les résultats sont rassemblés dans le Tableau I, la [2-^{14}C]-uracile a été ajoutée 45 min après l'azaguanine (36 μg/ml) à une suspension de bactéries en croissance; 1 h après l'addition de l'uracile, les bactéries ont été lavées dans un milieu contenant de l'uracile non marquée et de l'azaguanine, puis incubées soit en présence de guanosine soit en son absence dans ce même milieu. Il est clair (Tableau I) que les pyrimidines de l'ARN formé en présence d'azaguanine ne sont pas rejetées, alors que pendant ce temps l'azaguanine est expulsée de l'ARN dans lequel elle s'était incorporée (Tableau II).

TABLE I

EFFET DE LA GUANOSINE SUR L'URACILE INCORPORÉE EN PRÉSENCE D'AZAGUANINE

A une suspension de *B. cereus* (0.3 mg/ml) en croissance, on a ajouté de l'azaguanine (36 μg/ml) puis, 45 min plus tard (temps 0), de la [2-^{14}C]uracile (1.5 μg et 0.06 μC/ml). Entre la 65e et la 80e min, les bactéries ont été centrifugées, lavées deux fois à 30° avec du milieu de culture additionné d'uracile non radioactive et d'azaguanine (36 μg/ml), et finalement reprises dans un milieu de même composition. La suspension a été partagée en deux parties égales; à l'une d'elles on a ajouté de la guanosine (135 μg/ml), l'autre a servi de témoin. Des échantillons de 1 ml ont été prélevés et mélangés à une solution d'acide trichloracétique; les précipités ont été recueillis sur filtres "Millipore" et leur radioactivité a été mesurée.

Temps (min)	*Radioactivité du précipité coups/min/ml de suspension*	
0	0	
15	1711	
30	4284	
60	7810	
65 à 80	(lavages, partage)	
80	(0 μg guanosine)	(+ 135 μg guanosine/ml)
90	6407	6584
120	6652	6514
150	6990	7127
180	6831	7212
215	6811	7335
240	6529	7164

Chez *B. cereus*, les pyrimidines de l'ARN formé en présence d'azaguanine ne subissent donc pas le même sort que l'azaguanine contenue dans cet ARN quand on ajoute de la guanosine: les pyrimidines restent dans des polynucléotides tandis que l'azaguanine est expulsée et se retrouve dans le milieu sous forme d'azaguanosine[5]. Ceci pourrait indiquer un échange entre l'azaguanine des ARN et la guanosine libre sans que l'édifice polynucléotidique se défasse complètement. MATTHEWS ET SMITH[2] ont, les premiers, attiré l'attention sur une telle possibilité.

Cultivons *B. cereus* en présence de [8-^{14}C]guanine, puis transférons les bactéries marquées dans un milieu non radioactif, et ajoutons alors de l'azaguanine (36 μC/ml). Bien que l'action de l'azaguanine sur l'accroissement de la densité optique de la suspension se fasse sentir normalement, toute la guanine qui se trouvait dans l'ARN au début de l'expérience y reste (Tableau III); elle n'est aucunement déplacée par l'azaguanine. On ne peut donc pas dire que la guanine et l'azaguanine s'échangent.

TABLE II

EFFET DE LA GUANOSINE SUR L'AZAGUANINE PRÉALABLEMENT INCORPORÉE

La [2-^{14}C]azaguanine a été ajoutée (36 µg/ml) au temps 0 à une suspension de *B. cereus* en croissance. 65 min plus tard, la suspension a été partagée en deux parties égales. De la guanosine (135 µg/ml) a été ajoutée à l'une d'elles, l'autre n'a reçu aucune addition. Les chiffres indiquent la radioactivité du précipité trichloracétique par ml de suspension bactérienne. 1000 coups/min correspondent à 1.25 µg d'azaguanine.

Temps (min)	*Radioactivité du précipité coups/min/ml de suspension*	
0	4	
15	170	
30	400	
60	683	
65	(0 µg guanosine)	(+ 135 µg guanosine/ml)
90	950	541
120	1251	457
150	1572	360
180	1793	283
140	2050	230

TABLE III

EFFET DE L'AZAGUANINE SUR LA GUANINE PRÉALABLEMENT INCORPORÉE

Les bactéries ont été cultivées dans un milieu contenant 2 µmoles de [8-^{14}C]guanine (1 µC/µmole)/ml. Elles ont été récoltées après 18 h de culture, lavées et reprises dans du milieu de culture sans guanine, à 30°. La suspension de bactéries marquées a été partagée en deux parties égales; à l'une d'elles on a ajouté 36 µg d'azaguanine/ml, l'autre a été conservée comme témoin. Les chiffres ci-dessous indiquent la radioactivité du précipité trichloracétique d'échantillons (1 ml) de suspension bactérienne prélevés au cours de l'incubation.

Temps (min)	*Radioactivité du précipité Coups/min/ml de suspension*	
	0 µg azaguanine	*36 µg azaguanine/ml*
0	3703	3870
30	3575	3886
60	3696	3697
90	3663	3769
120	3882	3837
180	3545	3612

Le remplacement—si c'est de cela qu'il s'agit—ne se fait que dans un sens: l'acide guanylique se substitue peut-être à une partie de l'acide azaguanylique présent dans l'ARN anormal, mais l'acide azaguanylique ne se substitue pas au nucléotide normal dans les ARN normaux. Le remplacement est donc propre aux ARN anormaux; il n'est sans doute possible que parce que l'acide azaguanylique se trouve surtout en bout de chaîne[5]. Nos expériences n'excluent cependant pas un remaniement plus profond des ARN anormaux sous l'action de la guanosine.

Influence de l'azaguanine sur la stabilité chimique de l'ARN

Des irrégularités dans les résultats des dosages d'ARN ont attiré notre attention sur la labilité en milieu acide des ARN chez les bactéries qui ont subi l'action de l'azaguanine. Nous avons étudié systématiquement l'hydrolyse de l'ARN en milieu $HClO_4$ 3 % à 37°.

A une suspension de bactéries en croissance exponentielle, ajoutons 36 μg d'azaguanine/ml puis, 15 min plus tard, de la [2-^{14}C]uracile (0.1 μC et 10 μg/ml). Prélevons des échantillons de cette suspension 30, 60, 120 et 180 min plus tard, et mélangeons-les à un volume d'alcool, à 0°. Après lavage à l'alcool 70 %, les précipités seront remis en suspension dans de l'acide perchlorique à 3 % et maintenus à 37° en thermostat. Des échantillons prélevés au cours de l'incubation seront filtrés rapidement sur filtre "Millipore". Après lavage par de l'acide perchlorique froid, les précipités seront séchés et leur radioactivité mesurée.

La quantité de ^{14}C restant sur le filtre est une mesure approximative de la quantité d'ARN formé en présence d'azaguanine et non encore hydrolysé. (On néglige ici l'ADN qui contient environ 10 % du ^{14}C avant l'hydrolyse.)

Il est clair que les ARN formés en présence d'azaguanine sont labiles et qu'ils le sont d'autant plus que l'analogue a agi plus longtemps (Fig. 6a).

La même expérience a été faite en marquant les ARN formés avant l'action de l'azaguanine. Pour cela, *B. cereus* a été cultivé dans un milieu contenant 7 μg et 0.03 μC de [2-^{14}C]uracile/ml, transféré dans un milieu non radioactif où il a été cultivé encore pendant 2 h. Ensuite les bactéries ont été lavées et reprises dans un milieu frais contenant de l'uracile non radioactive. La suspension a été partagée en deux; l'une des partie a été additionnée de 36 μg d'azaguanine/ml, l'autre a servi de témoin. On a

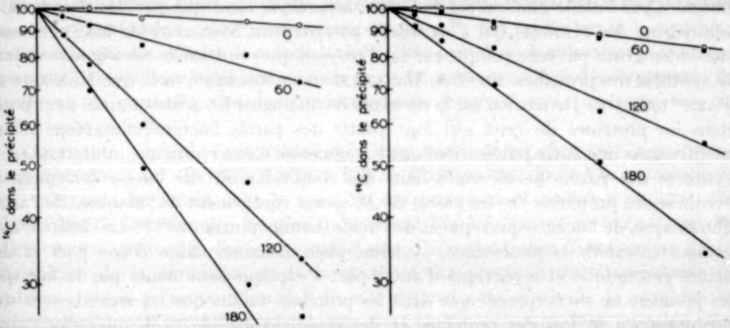

Fig. 6. Labilisation des ARN pendant l'incorporation d'azaguanine. Courbes d'hydrolyse dans $HClO_4$ 3 % à 37° des acides nucléiques de bactéries prélevées à différents temps après l'addition d'azaguanine (36 μg/ml). Les chiffres inscrits auprès des courbes indiquent la durée d'incubation des bactéries en présence d'azaguanine. (a) Courbes d'hydrolyse des acides nucléiques formés en présence d'azaguanine. (b) Courbes d'hydrolyse des acides nucléiques qui s'étaient formés avant l'addition de l'azaguanine (voir texte).

étudié comme précédemment l'hydrolyse de l'ARN marqué (c'est à dire dans le cas présent l'ARN formé en l'absence d'azaguanine). La Fig. 6b montre que l'azaguanine provoque une certaine labilisation des ARN qui s'étaient formés dans les bactéries avant l'addition de l'analogue. Mais l'effet est beaucoup plus tardif que pour les ARN formés en présence de l'azaguanine.

Il ne semble pas que ces modifications des propriétés des ARN puissent s'expliquer par la présence, l'activation ou la formation d'enzymes nucléolytiques. En effet, nous n'avons pu détecter aucune activité de ce type en effectuant des dosages de "ribonucléase" dans des extraits de bactéries broyées à l'alumine. La recherche d'enzyme a été effectuée à pH 5 et 7, par les méthodes de CHANTRENNE[28], de KUNITZ[27] et de DICKMAN[30]. Les dosages n'ont révélé que des traces d'activité nucléasique qui n'étaient d'ailleurs pas modifiées par le traitement préalable des bactéries par l'azaguanine.

DISCUSSION

Nos résultats montrent clairement que la synthèse des protéines est fortement inhibée par l'azagunine. Que l'on dose les substances protéiques par la méthode de Lowry, ou que l'on mesure l'incorporation de méthionine, de phénylalanine, de leucine ou de valine dans les substances précipitées par l'acide trichloracétique, on arrive à la même conclusion. MANDEL a également observé que l'incorporation des acides aminés contenant du soufre est fortement inhibée[19]. Toutefois l'incorporation de l'acide glutamique dans le précipité trichloracétique de même que la synthèse des substances dosées par la ninhydrine n'est pas inhibée; pour un accroissement donné de la densité optique, elle est même accrue[31]. MANDEL pense que l'analogue modifierait les proportions des différents acides aminés qui s'incorporent dans les protéines. Nous avons obtenu pour l'acide glutamique et l'acide aspartique ainsi que par des dosages à la ninhydrine des résultats qui s'accordent parfaitement avec ceux de MANDEL, mais nous n'en avons pas tenu compte car nous croyons que ces données renseignent mal sur la synthèse des protéines. En effet, MANDELSTAM ET ROGERS[17], ainsi que HANCOCK ET PARK[18] ont attiré l'attention sur la nécessité de distinguer les acides aminés incorporés dans les protéines de ceux qui font partie des parois bactériennes. Nous avons montré dans une autre publication[8] que l'azaguanine n'exerce aucune inhibition sur la synthèse des parois bactériennes dans des conditions où elle inhibe fortement la synthèse des protéines. Or, les parois de *B. cereus* contiennent de l'alanine, de l'acide glutamique, de l'acide aspartique et de l'acide diaminopimélique[15, 16]. La différence de comportement de la méthionine, cystéine, phénylalanine, valine d'une part et des acides glutamique et aspartique d'autre part s'explique sans doute par le fait que les premiers ne s'incorporent que dans les protéines tandis que les seconds sont des précurseurs à la fois des protéines et des constituants non protéiques des parois bactériennes, dont la formation n'est pas affectée par l'azaguanine.

D'ailleurs, RICHMOND[32], qui a étudié l'effet de plusieurs toxiques sur *B. subtilis*, bactérie très étroitement apparentée à *B. cereus*, a établi que l'azaguanine inhibe fortement l'incorporation d'alanine dans les protéines, sans affecter l'incorporation du même acide aminé dans les parois bactériennes isolées par un traitement limité au lysozyme.

Nous croyons aussi que la comparaison de la densité optique des suspensions de bacteries traitées par l'azaguanine à celle de bactéries normales peut induire en erreur.

En effet, l'analogue modifie profondément les vitesses relatives de synthèse des protéines, ARN, ADN et parois bactériennes[8]; aussi la composition des bactéries intoxiquées est-elle toute différente de celle des bactéries témoins. La quantité de constituants des parois bactériennes est fortement accrue par rapport aux protéines, et la densité optique n'a pas la même signification chez les bactéries qui ont subi l'action de l'azaguanine que chez les témoins. C'est la raison pour laquelle nos résultats n'ont pas été rapportés à la densité optique des suspensions.

Ces remarques n'excluent pas que l'idée de MANDEL puisse être en partie correcte. La faible synthèse de protéines qui se poursuit en présence d'azaguanine pourrait correspondre à la formation de protéines anormales. L'inhibition complète de la synthèse de pénicillinase dans des conditions où l'incorporation de phénylalanine n'est pas complètement supprimée (Fig. 3) ainsi que d'autres phénomènes qui accompagnent le rétablissement de la synthèse protéique après inhibition par l'azaguanine[33–35], s'accordent en effet avec l'idée que l'azaguanine agirait au niveau d'un mécanisme qui régit la spécificité des synthèses protéiques.

Quoiqu'il en soit, les résultats de MANDEL et les nôtres s'accordent pour montrer que la synthèse de protéines parfaites—sinon celle de substances polypeptidiques—est supprimée peu de temps après l'addition de l'azaguanine. Il en résulte que le contenu en enzymes par ml de suspension bactérienne cesse d'augmenter quelques minutes après l'addition de l'analogue tandis qu'il s'accroît au rythme de la croissance chez les bactéries normales. On peut prévoir que les synthèses deviendront tôt ou tard linéaires dans une suspension de bactéries intoxiquées par l'azaguanine, exactement comme cela se produit chez des bactéries soumises à l'action d'analogues d'acides aminés[36].

Plusieurs observations indiquent que l'azaguanine peut inhiber la synthèse des protéines dans divers types de cellules, par exemple *Bacillus subtilis*[32, 41], *Staphylococcus aureus*[38], *Tetrahymena geleii*[39], les racines d'oignons[39]; elle inhibe aussi la synthèse d'anticorps par des cellules de rate de lapin[40]. C'est peut-être en entravant la synthèse des protéines qu'elle ralentit la croissance de certaines tumeurs[46], le développement de l'embryon de poulet[47], celui du gamétophyte de fougère[44] et la multiplication de bactériophages[37, 45].

La nature même de l'azaguanine et la suppression de ses effets par la guanosine nous renseignent sur son mode d'action. Elle se substitue sans doute à la guanine dans une substance impliquée dans la synthèse des protéines. MANDEL[4] a souligné que l'inhibition de la croissance est plus directement liée à la teneur en azaguanine des acidosolubles qu'à celle des acides nucléiques. Cependant chez *Tetrahymena geleii*, l'inhibition de la croissance et de la synthèse des protéines par l'azaguanine ne s'observe qu'en présence d'uracile[39, 43]; elle dépend sans doute de la synthèse de polynucléotides. Il est donc probable que c'est en s'incorporant dans un acide ribonucléique particulier, en liaison métabolique assez étroite avec les acidosolubles, que l'azaguanine inhibe la synthèse des protéines.

Cela n'exclut nullement une action de l'azaguanine sur d'autres ARN, plus inertes, voire sur l'ADN.

Les effets de faibles concentrations d'azaguanine (3 à 4 μg/ml) sur la formation des protéines et des ARN sont qualitativement semblables à ceux de concentrations 10 fois plus fortes. Par contre, la synthèse d'ADN n'est inhibée que si la concentration de l'analogue est élevée. Il en résulte qu'on peut dissocier ces effets.

En présence de 3.6 μg d'azaguanine/ml, concentration qui suffit à supprimer la synthèse d'enzymes actifs—et peut-être celle de toute protéine normale—l'ADN peut effectuer plusieurs duplications. Nous ignorons si l'ADN qui se forme dans ces conditions est normal; mais puisqu'il s'accroît au même rythme que chez les bactéries témoins, il faut en conclure qu'il conserve au moins la capacité d'accomplir la fonction qui lui revient dans sa propre multiplication.

Au contraire, si la concentration de l'azaguanine est 10 fois plus élevée, la vitesse de synthèse de l'ADN est légèrement réduite dès le début, et elle diminue fortement vers le moment où la quantité d'ADN a doublé. Tout se passe comme si l'ADN formé dans ces conditions perdait, dans une large mesure, la faculté de se multiplier. Cela s'explique peut-être par l'incorporation de faibles quantités d'azaguanine aux chaînes d'ADN formées en présence de concentrations élevées de l'analogue.

Soulignons enfin que les effets de l'azaguanine sur *B. cereus* sont très semblables à ceux de la chloromycétine: inhibition de la synthèse des protéines, accumulation d'ARN anormaux, inhibition tardive de la synthèse des ADN, absence d'action sur la formation des parois cellulaires. Il est vraisemblable que les deux toxiques agissent à peu près au même niveau sur le mécanisme de ces synthèses.

RÉSUMÉ

L'azaguanine inhibe fortement la synthèse des protéines et elle supprime complètement la formation de pénicillinase constitutive, chez *B. cereus*.

De faibles concentrations d'azaguanine suffisantes pour supprimer la synthèse de pénicillinase ne modifient pas la vitesse de synthèse de l'ADN. Des concentrations plus élevées exercent sur la synthèse de l'ADN une certaine inhibition qui s'accentue vers le moment où l'ADN a doublé.

L'azaguanine stimule la synthèse des ARN. Lorsque la concentration de l'azaguanine est faible, l'analogue ne s'incorpore dans les ARN que pendant quelque temps; une grande partie de l'azaguanine contenue dans les ARN est ensuite éliminée.

L'ARN de bactéries qui ont incorporé de l'azaguanine est plus labile en milieu acid que l'ARN normal.

L'action de l'azaguanine sur *B. cereus* ressemble fort à celle de la chloromycétine sur *E. coli* ou sur *S. aureus*.

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REPLACEABILITY OF THE pH 5 ENZYMES FRACTION OF RAT LIVER BY THE AMINO ACID INCORPORATION FACTOR OF *STAPHYLOCOCCUS AUREUS*[*]

S. R. WAGLE[**], RANJAN MEHTA[**] AND B. CONNOR JOHNSON
Department of Animal Science, University of Illinois, Urbana, Ill. (U.S.A.)

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SUMMARY

It was found using a liver microsome system from normal rats that the *in vitro* incorporation of glycine or alanine into microsomal protein could be brought about by the addition of either pH 5 enzymes, (or a fraction of it) or Gale's incorporation factor. The activity of pH 5 enzymes and incorporation factor appeared to be mutually replaceable.

INTRODUCTION

By digesting *Staphylococus aureus* RNA with RNase. Gale and Folkes[1,2] isolated a factor which they have termed "incorporation factor" and which stimulates the incorporation of amino acids into a *S. aureus* ruptured cell system. These investigators have reported the inhibition of their system by various benzimidazoles and thus it was of interest to us to study the effect of their incorporation factor on amino acid incorporation into protein in the rat liver pH 5 enzymes-microsomes system, because of the effect of the benzimidazole compound, vitamin B_{12} in this latter system[3]. The incorporation factor for this study was generously provided by Dr. E. F. Gale of Cambridge, England.

The work of Hoagland *et al.*[4] has revealed that pH 5 enzymes are essential for the activation of amino acids before incorporation into protein. Work from this laboratory[3] indicated that a fraction of pH 5 enzymes precipitated at 20–60 % saturation ammonium sulfate contained all the activity and stimulated the incorporation of amino acid into protein by two fold. The present investigations are concerned with the relationship of pH 5 enzymes and Gale's incorporation factor activity in an animal system.

Abbreviations used: RNA, ribonucleic acid; RNase, ribonuclease; TCA, trichloroacetic acid; ATP, adenosine triphosphate; AMP, adenosine monophosphate; GIF, Gale's incorporation factor; GTP, guanosine triphosphate; ASF, ammonium sulfate fraction.

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[**] Present address: Haffkine Institute, Parel, Bombay 12, India.

EXPERIMENTAL

Preparation of microsomes and pH 5 enzymes

Approx. 50 g of normal rat liver were homogenized, 10 g at a time with 2.5 times its volume of buffered medium as described previously[3]. The microsomes and pH 5 enzymes were then prepared by centrifugation as reported earlier[3].

Ammonium sulfate fractionation of pH 5 enzymes

Preparation of supernatant: 250 g of normal rat liver obtained fresh from rats killed by decapitation were chilled in ice and homogenized in 750 ml of ice cold buffered medium[3] using a Waring blendor. All steps in these studies were carried out at 0°. Homogenization was done at full speed for 1 min. The homogenate was centrifuged for 15 min. at 15,000 × *g* in a refrigerated centrifuge (Servall SS-1 Model). The supernatant obtained was passed through several layers of cheesecloth to remove fat. The supernatant was then centrifuged at 105,000 × *g* for 1 h. The microsomes were discarded and the supernatant was again passed through cheesecloth for removal of residual fat to give supernatant I. This supernatant I was adjusted to pH 5.2 with *N* acetic acid. The precipitate thus obtained was centrifuged down at 10,000 × *g* and redissolved in 300 ml of buffered medium.

Removal of nucleic acids: 3 ml of 1% protamine sulfate solution were added drop by drop to a constantly stirred solution. The solution was allowed to stand for 1 h and then centrifuged at 10,000 × *g* for 10 min. to give supernatant II. The precipitated nucleic acids were discarded.

Preparation of 20–60 % saturation ammonium sulfate fraction: To 300 ml of supernatant II solid ammonium sulfate was added to give 20 % saturation. After 3 h the precipitate was removed and discarded. The supernatant fluid was then brought to 60 % saturation to give a 20–60 % ammonium sulfate fraction precipitate. The precipitate was obtained by centrifugation and was dissolved in buffered medium and dialyzed for 24 h against 1,000 ml of the buffered medium with three changes. The fraction was then used in the incorporation system.

Stability of incorporation factor activity

The stability of incorporation factor activity to 1 *N* acid and alkali was carried out as described by GALE *et al.*[1,2].

Preparation of nucleic acids

A large amount of pH 5 enzymes was precipitated as above from the supernatant from normal rat livers. The precipitate was heated with 0.5 % NaCl at 50° and undissolved material was removed by centrifugation. The supernatant fluid was cooled to 0° and previously cooled (— 10°) 95 % ethanol (3 volumes) was added to precipitate the nucleic acids. The precipitate was dissolved in buffered medium and treated with amylase at 37° for 3 h. The solution was then treated with 3 volumes of ethanol and the precipitate obtained was dissolved in cold distilled water and dialyzed for 24 h against distilled water to remove any traces of NaCl. The concentration of nucleic acid in this solution was approx. 5 mg/ml. This solution was stored frozen at — 20° and used as source of supernatant nucleic acids.

Incorporation of amino acid into protein was then carried out as described in each table. The mixtures were incubated (under N_2 containing 5 % CO_2) for 1 h at 37°.

After incubation, proteins were precipitated with 10 % TCA, washed and assayed for ^{14}C activity as described previously[5].

RESULTS AND DISCUSSION

The results in Table I show that the addition of GIF to microsomes in the absence of pH 5 enzymes gave marked stimulation of amino acid incorporation equivalent to that given by the 20–60 % ASF. From Table II some stimulation can be seen for glycine in the presence of pH 5 enzyme at the level used; however, when pH 5 enzyme was replaced by the 20–60 % ASF, then no further stimulation was brought about by GIF addition. The results demonstrate the mutual replaceability of the active com-

TABLE I

EFFECT OF GALE'S INCORPORATION FACTOR (GIF) IN ABSENCE OF pH 5 ENZYMES ON INCORPORATION OF [2-^{14}C]GLYCINE AND [2-^{14}C]ALANINE INTO LIVER MICROSOMAL PROTEIN

Complete system contained 0.3 ml microsomes (7.5 mg protein), 0.25 μmole [2-^{14}C]glycine, or [2-^{14}C]alanine, 10 μmole phosphoenol pyruvate, 0.02 mg pyruvate kinase, 0.5 μmole ATP, 0.25 μmole GTP, final volume made to 1.0 ml by addition of 0.15 *M* KCl. Incubation mixtures 3–7 also contained 0.5 mg of RNA.

Added	*[2-^{14}C]glycine incorporation counts/min/mg protein*	*[2-^{14}C]alanine incorporation counts/min/mg protein*
1. Complete system	20	32
2. Complete system + pH 5 enzymes (2.0 mg)	68	98
3. Complete system + 20–60* fraction (2.0 mg)	112	182
4. Complete system + GIF 0.5 units	122	188
5. Complete system + GIF 1.0 units	120	176
6. Complete system + GIF 2.0 units	128	166
7. Complete system + GIF 5.0 units	102	130
8. Complete system + GIF (2 units treated with 1 *N* NaOH for 1 h)	108	—
9. Complete system + GIF (2 units treated with 1 *N* HCl for 1 h)	29	—

* 20–60 fraction = fraction of pH 5 enzymes precipitated by 20–60 % saturation with ammonium sulfate.

TABLE II

EFFECT OF GALE'S INCORPORATION FACTOR (GIF) IN PRESENCE OF pH 5 ENZYMES ON INCORPORATION OF [2-^{14}C]GLYCINE AND [2-^{14}C]ALANINE INTO LIVER MICROSOMAL PROTEIN

Complete system contained 0.3 ml microsomes (7.5 mg protein), 0.25 μmole [2-^{14}C]glycine or [2-^{14}C]alanine, 10 μmoles phosphoenol pyruvate, 0.02 mg pyruvate kinase, 0.5 μmole GTP, final volume made to 1.0 ml by addition of 0.15 *M* KCl. Incubation mixtures 4–8 also contained 0.5 mg of RNA.

Added	*[2-^{14}C]glycine incorporation counts/min/mg protein*	*[2-^{14}C]alanine incorporation counts/min/mg protein*
1. Complete system	15	20
2. Complete system + pH 5 enzymes (2.0 mg)	62	78
3. Complete system + pH 5 enzymes + GIF 2.0 units	130	88
4. Complete system + 20–60* fraction (2.0 mg)	112	182
5. Complete system + 20–60 fraction + GIF 0.5 units	108	118
6. Complete system + 20–60 fraction + GIF 1.0 units	102	124
7. Complete system + 20–60 fraction + GIF 2.0 units	112	130
8. Complete system + 20–60 fraction + GIF 5.0 units	86	80

* 20–60 fraction = fraction of pH 5 enzymes precipitated by 20–60 % saturation with ammonium sulfate.

ponent of the pH 5 enzymes, and GIF, and would suggest the presence of similar active components in GIF and 20–60 % ASF.

The activities in the two preparations appear to be replaceable—where one is omitted—and additive—where pH 5 enzyme is added in suboptimal amount then GIF gives increased incorporation. The differences in amount of incorporating activity for glycine and alanine in the pH 5 enzyme preparation, as evidenced by the additional effect of GIF for glycine and not for alanine, suggest the presence of individual enzymes for each amino acid.

The stability of the material in the incorporation factor concentrate active in the rat system, to alkali treatment and lability to acid treatment, as shown in Table I, is the same as that reported by GALE *et al.*[1,2] for the material active in the bacterial system giving a further indication that the same factor in these preparations is responsible for activity in both systems.

The results reported here may be similar to those reported by BELJANSKI AND OCHOA[6]. They reported that they could replace pH 5 enzymes of rat liver by the amino acid incorporation enzyme obtained from *Alcaligenes faecalis*. Thus both our data[7] and theirs[6] indicate that incorporation factors of bacterial origin can replace pH 5 enzymes of rat liver. Recent studies of GALE *et al.*[8] suggest that the incorporation factor is involved in the synthesis of ribonucleic acid. However, studies carried out recently show that GIF does not increase the incorporation of [^{14}C]AMP or [^{14}C]ATP into RNA[9] when added to rat liver pH 5 enzymes microsome system. Also the addition of RNA is required along with microsomes with GIF as with pH 5 enzymes for amino acid incorporation by the rat liver system[3].

An important advance in the understanding of the mechanism of protein synthesis was the finding by HOAGLAND *et al.*[4] of an ATP amino acid activation step linked with pyrophosphate exchange. The fact that pH 5 enzymes can be replaced by the incorporation factor suggests either the same active component or possibly an alternate pathway for the incorporation of amino acid into protein without pH 5 enzymes. Further studies are now in progress to investigate the role of incorporation factor into protein and nucleic acid synthesis in the animal system.

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RIBONUCLEIC ACID SYNTHESIS IN MICROSOMES AND pH 5 ENZYMES*

RANJAN MEHTA**, S. R. WAGLE AND B. CONNOR JOHNSON

Department of Animal Science, University of Illinois, Urbana, Ill. (U.S.A.)

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SUMMARY

Incorporation of [^{14}C]AMP and [^{14}C]ATP into RNA has been studied using a rat-liver enzyme-microsome system. Both the nucleotides were incorporated to the same extent. The incorporation was increased in the presence of a mixture of amino acids, a mixture of mononucleotides or a mixture of trinucleotides.

INTRODUCTION

The addition of adenine nucleotides as the terminal groups of RNA has been observed in several laboratories[1-4] and evidence has been presented in favour of ATP as the precursor rather than ADP. The recent work by HECHT *et al.*[5] suggests the end group of ribonucleic acid as two cytosine molecules linked to a terminal adenosine molecule. It has been shown that RNA of pH 5 enzymes plays an important role as an acceptor of the activated amino acids in protein synthesis[6-9]. The present investigation was carried out to study the effect of various conditions which affect protein biosynthesis, on RNA synthesis, as it appears that these two are simultaneous and interdependent.

EXPERIMENTAL

Incorporation of [^{14}C]AMP into RNA of liver homogenates free from nuclei, debris and mitochondria

Rats were killed by decapitation and the livers removed and immediately transferred to the cold homogenization medium described previously[10]. An homogenate free from mitochondria, nuclei and cell debris was obtained by centrifuging the whole homogenate at 800 × *g* for 10 min. The supernatant obtained after centrifugation was incubated with AMP alone or with AMP, UMP, CMP and GMP; in the presence of GALE's incorporation factor, and also in the presence of one or a mixture of amino

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Abbreviations used: RNA, ribonucleic acid; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CTP, cytosine triphosphate; UMP, uridine monophosphate; UTP, uridine triphosphate; PEP, phosphoenol pyruvate; PK, pyruvate kinase; GIF, GALE's incorporation factor.

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** Present address: Haffkine Institute, Parel, Bombay 12, India.

acids. The incubation mixtures were supplemented with PEP, PK, and $MgCl_2$ and the incubations were carried out under N_2 (containing 5 % CO_2) for 1 h at 37°. At the end of the incubation period the incubation mixtures were heated to 100° and RNA was isolated as follows: 100 mg of NaCl was added to each mixture and they were heated at 70° in a water bath for 1 h. At the end of the hour the solutions were cooled and centrifuged. To each supernatant 3 volumes of 95 % ethanol was added and stirred vigorously. The precipitate was collected by centrifugation and redissolved in distilled water and reprecipitated with 3 volumes of 95 % ethanol. This was repeated twice. It was then redissolved in distilled water and treated with amylase and incubated for 2 h at 37°. After the incubation 3 volumes of 95 % ethanol were added and the precipitate collected by centrifugation. It was then dissolved in water and the pH was adjusted to 4.2 with 0.1 *N* HCl. The precipitate obtained was discarded and to the supernatant was added 3 volumes of 95 % ethanol after which the precipitate obtained was washed with hot alcohol followed by an acetone–ether mixture (1:1). The RNA thus obtained was dissolved in distilled water and counted as described previously[11]. The results of this study are given in Table I.

TABLE I

RNA SYNTHESIS IN HOMOGENATE FREE FROM NUCLEI AND MITOCHONDRIA

Complete system contained 2.0 ml homogenate (40 mg protein), 2.0 µmoles ATP, 20 µmoles PEP, and 0.1 mg PK; 0.5 µmole $MgCl_2$. AMP, GMP, UMP and CMP were added in equimolar concentrations (2.0 µmoles). Phenylalanine added at 0.25 µmole; mixture of amino acids — 20 amino acids added at 0.25 µmole each; GIF, 2 units. Total volume 5 ml with 0.15 *M* KCl.

Added	*Total counts/min in RNA*
1. Complete system + [^{14}C]AMP alone	230
2. Complete system + [^{14}C]AMP, GMP, UMP, CMP	780
3. Complete system + [^{14}C]AMP + amino acid mixture	466
4. Complete system + [^{14}C]AMP + phenylalanine	235
5. Complete system + [^{14}C]AMP, GMP, UMP, CMP + phenylalanine	740
6. Complete system + [^{14}C]AMP, GMP, UMP, CMP + amino acid mixture	830
7. Complete system + [^{14}C]AMP, GMP, UMP, CMP + amino acid mixture + GIF*	760
8. Complete system + [^{14}C]AMP, GMP, UMP, CMP + GIF	676
9. Complete system + [^{14}C]AMP, GMP, UMP, CMP + GIF + phenylalanine	720

* We are grateful to Dr. E. F. GALE for the incorporation factor.

The incorporation of [^{14}C]AMP into RNA of pH 5 enzymes and microsomes by a rat-liver system

Preparation of microsomes and pH 5 enzymes was carried out as described previously[10]. The pH 5 enzymes and microsomes were incubated with AMP alone or with AMP, UMP, CMP and GMP; in the presence of a single or a mixture of amino acids and also in the presence of GALE's incorporation factor. Incubation and addition of other cofactors was similar to the previous experiment. Isolation of RNA was carried out as described above. The results of this study are given in Table II.

The incorporation of ATP as compared to the incorporation of AMP into RNA

Experimental conditions were similar to those described above, but this time the effect of a mixture of ATP, CTP, UTP and GTP was also studied. The results are given in Table III.

TABLE II

RNA SYNTHESIS IN pH 5 ENZYMES-MICROSOMES SYSTEM

Complete system contains 2.0 ml of pH 5 enzyme (5 mg protein) and microsomes (15 mg protein); 2.0 μmoles ATP, 20 μmoles PEP, 0.1 mg PK; 0.5 μmole $MgCl^2$; [^{14}C]AMP, GMP, UMP and CMP were added in equimolar concentrations (2.0 μmoles). Phenylalanine added at 0.25 μmole; mixture of amino acids — 20 amino acids added at 0.25 μmole each; GIF, 2 units.

Added	*Total counts/min in RNA*
1. Complete system + [^{14}C]AMP alone	260
2. Complete system + [^{14}C]AMP, GMP, UMP, CMP	820
3. Complete system + [^{14}C]AMP + amino acid mixture	510
4. Complete system + [^{14}C]AMP, GMP, UMP, CMP + phenylalanine	785
5. Complete system + [^{14}C]AMP + phenylalanine	250
6. Complete system + [^{14}C]AMP, GMP, UMP, CMP + amino acid mixture	960
7. Complete system + [^{14}C]AMP, GMP, UMP, CMP + amino acid mixture + GIF	785
8. Complete system + [^{14}C]AMP, GMP, UMP, CMP + GIF	880
9. Complete system + [^{14}C]AMP, GMP, UMP, CMP + GIF + phenylalanine	830

TABLE III

THE INCORPORATION OF [^{14}C]AMP AND [^{14}C]ATP INTO RNA OF pH 5 ENZYMES-MICROSOMES SYSTEM

Complete system contained 2.0 ml of pH 5 enzymes (10 mg protein) and microsomes (30 mg protein); 2.0 μmoles ATP, 20 μmoles PEP, 0.1 mg PK, 0.5 μmole $MgCl_2$; [^{14}C]AMP, UMP, CMP, GMP and ATP, GTP, UTP, CTP were added in equimolar concentrations (2.0 μmoles). Phenylalanine added at 0.25 μmole; mixture of amino acids — 20 amino acids added at 0.25 μmole each; GIF, 2 units.

Added	*Total counts/min in RNA*	
	[^{14}C]AMP	*[^{14}C]ATP*
Complete system	550	540
Complete system + phenylalanine	490	405
Complete system + amino acid mixture (20)	1,410	1,430
Complete system + AMP (cold)	485	400
Complete system + ATP, GTP, UTP, CTP	3,000	2,840
Complete system + AMP, GMP, UMP, CMP	2,900	3,100
Microsomes + GIF, 2 units	615	762
Microsomes + Amino acid mix. + GIF	1,350	1,400
Microsomes + AMP, UMP, CMP, GMP + GIF	2,100	1,900
Microsomes + ATP, UTP, GTP, CTP + GIF	3,140	2,500

RESULTS AND DISCUSSION

The data presented in Tables I, II, and III show that a system has been found for the *in vitro* incorporation of [^{14}C]AMP and [^{14}C]ATP into the RNA of pH 5 enzymes and microsomes. It is evident from the data that the factors which affect general protein biosynthesis or at least amino acid incorporation into protein also affects the incorporation of [^{14}C]AMP and [^{14}C]ATP into RNA. For example, a mixture of amino acids increased the incorporation of nucleotides almost two fold, as it does in the case of the incorporation of amino acids into protein[12]. Moreover a mixture of mononucleotides and trinucleotides increased the incorporation of both [^{14}C]AMP and [^{14}C]ATP about four-fold.

Hecht *et al.*[5] have also found that there is increased incorporation of [^{14}C]AMP into RNA in the presence of a mixture of nucleotides.

An interesting observation (Table III) is that pH 5 enzymes can be replaced by GIF. It can also be seen that when GIF is used along with pH 5 enzymes, the effects of the two are not supplementary or additive, and it thus appears that they serve the same purpose. This agrees very well with the data on amino acid incorporation and the replaceability of GIF and pH 5 enzymes[13]. Thus it seems very likely that pH 5 enzymes and GIF may have similar properties.

The data presented indicate the position of the occurrence of synthesis of RNA and protein simultaneously in pH 5 enzymes and microsomes and support the hypothesis suggested by Hecht *et al.*[5]:

$$\text{CTP} + \text{RNA} \rightleftharpoons \text{CMP-RNA} + \text{PP}$$

$$\text{ATP} + \text{CMP-RNA} \rightleftharpoons \text{AMP-CMP-RNA} + \text{PP}$$

$$\text{UTP} + \text{RNA} \rightleftharpoons \text{UMP-RNA} + \text{PP}$$

This hypothesis seems more convincing than the one previously suggested[14] that ADP rather than ATP is the precursor of RNA in this reaction[15], since the data show that both ATP and AMP are incorporated in the same proportion.

ACKNOWLEDGEMENTS

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A STUDY OF THE GENETIC MATERIAL DETERMINING AN ENZYME ACTIVITY IN *PNEUMOCOCCUS*

SANFORD LACKS* AND ROLLIN D. HOTCHKISS

The Rockefeller Institute, New York, N.Y. (U.S.A.)

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SUMMARY

An enzyme of *Pneumococcus* responsible for the conversion of maltose to glucose and oligosaccharides was investigated. The genetic analysis of a group of mutants lacking this enzyme was accomplished by studying DNA-mediated transformations of the different strains.

Transformation of the different mutants to maltose utilization by wild-type DNA occurred with different frequencies. Recombination of genetic material of the maltose-negative strains also gave rise to positive cells. Analysis of the recombinations indicated that the regions corresponding to the different markers are linked and arranged in a linear fashion. Some larger regions overlapped several smaller ones indicating that single mutations altered regions in the genetic material of different size.

Heating the DNA used in transforming the mutant strains destroyed most rapidly the ability to transform the factors corresponding to the larger genetic regions. Factors corresponding to smaller regions were inactivated less rapidly. This is taken to indicate that a DNA molecule partly denatured by heat still retains part of its genetic activity.

It is concluded, furthermore, that genetic properties of markers, such as size, linkage, and discreteness, are reflections of the actual distribution of determinants within DNA molecules.

INTRODUCTION

Genetic modification of pneumococcal cells by deoxyribonucleic acid from cells of different character has been described in a number of cases. In certain instances some limited definition could be made of the enzymic property affected by the DNA. Thus, transfers of the ability to utilize salicin[1], to oxidise lactate[2], to produce the inducible enzyme mannitol-phosphate dehydrogenase[3], and modifications in an enzyme system utilizing *p*-aminobenzoate for the synthesis of folic acid[4] have been reported.

Abbreviations: ATP, adenosine triphosphate; DNA, deoxyribonucleate; DNase, deoxyribonuclease; glucose-6-P, glucose-6-phosphate; TPN, triphosphopyridine nucleotide; Tris, tris-(hydroxymethyl)aminomethane.

[*] The work reported is to be submitted by this author as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The present study deals with the transfer of the inherited ability to produce an enzyme which can split maltose and hence enable the cells to grow in the presence of maltose as sole energy source. This transfer was made to cells of maltose-negative strains recovered after u.v. irradiation of the wild-type, positive strain. The genetic material involved in different mutations was examined both as to genetic relationships and physico-chemical nature.

EXPERIMENTAL TECHNIQUES

Two different media were used in the experiments. A glucose medium similar to that proposed by Adams and Roe[5] contained the following ingredients per liter: 5 g casein hydrolysate (Difco vitamin-free Casamino Acids), 6 mg tryptophane, 35 mg cystine, 2 g sodium acetate, 8.5 g K_2HPO_4, 0.5 g $MgCl_2 \cdot 6H_2O$, 2.5 mg $CaCl_2$, 25 μg $MnSO_4 \cdot 4H_2O$, 0.5 μg $FeSO_4 \cdot 7H_2O$, 0.5 μg $CuSO_4 \cdot 5H_2O$, 0.5 μg $ZnSO_4 \cdot 7H_2O$, 0.2 μg biotin, 0.2 mg nicotinic acid, 0.2 mg pyridoxine-HCl, 0.2 mg thiamine-HCl, 0.1 mg riboflavin, 0.6 mg calcium pantothenate, 2 g glucose, 12 ml 4 % bovine serum albumin, and 30 ml fresh yeast extract. The maltose medium was the same except that 2 g maltose were substituted for the glucose and yeast extract was replaced by 10 mg glutamine and 5 mg adenine.

Wild-type *pneumococcus* contains the maltase enzyme and is able to grow in the maltose medium. Mutant strains lacking these properties were obtained using the methods devised by Lederberg and Davis[6]. The procedure was as follows. A culture of *pneumococcus*, strain R6, was grown in glucose medium at 37° for 8 h. After transfer of 10 ml of the culture to a petri dish, the cells were irradiated with u.v. light from a GE Sterilamp so that about 10^{-3} of the cells survived. The irradiated culture was diluted tenfold in fresh glucose medium and incubated at 37° for 12 h. Then the cells were centrifuged, washed twice, and resuspended in an equal volume of maltose medium. After incubation at 37° for 30 min, penicillin was added to give a concentration of 0.2 units/ml, and incubation was continued for 18 h. About 50 % of the cells that survived this treatment were unable to utilise maltose. The eight mutant strains investigated were progeny of single colony isolates obtained from independently irradiated cultures.

Maltase activity was measured by a procedure in which the glucose released by the enzyme activity was converted to glucose-6-P by ATP in the presence of hexokinase: the resulting glucose-6-P in the presence of glucose-6-P dehydrogenase reduced TPN^+ to TPNH, the absorption of which was read at 340 mμ in a Beckman spectrophotometer. Enzyme activity was determined in lysates of the pneumococci prepared by adding 0.1 ml 1 % sodium deoxycholate to a suspension of 10^8 cells in 0.9 ml Tris buffer, 0.05 M, pH 7.2, containing 0.1 % glutathione and 0.5 % NaCl. Lysis occurred within 10 min on incubation at 37° after which cell debris was removed by centrifugation. A 0.1-ml aliquot of the lysate was added to a mixture of the following reagents contained in a 1-ml capacity Beckman cell: 0.6 ml Tris buffer pH 7.2, 0.05 M; $MgSO_4 \cdot 7H_2O$, $4 \cdot 10^{-3}$ M; ATP, $2 \cdot 10^{-3}$ M; TPN^+, $4 \cdot 10^{-4}$ M; hexokinase, Sigma, 25 μg; glucose-6-P dehydrogenase, Sigma, 0.008 units; glutathione, 0.1 %; maltose (twice recrystallized), 2 %. The cell was incubated at 37° and the increase in absorption at 340 mμ was measured.

Analysis of the products of the enzyme action on maltose was accomplished by ascending paper chromatography on Whatman No. 3 paper using *n*-propanol–ethyl acetate–water (6:1:3) as solvent and aniline–diphenylamine as detecting agent[7]. To 0.5 ml samples of a wild-type pneumococcal lysate were added sodium fluoride and maltose to give final concentrations of 0.1% and 2%, respectively. These were incubated for various lengths of time at 37° after which the reaction was arrested by heating 5 min at 100°, and after deionization with ion-exchange resins, 0.05-ml aliquots were spotted on the paper.

Transformations were performed as follows. Mutant cells were grown to a density of about 10^7/ml and then frozen in the presence of 10% glycerol[8]. A sample of such a culture was thawed and diluted one hundred-fold in fresh glucose medium. The DNA, prepared according to McCarty and Avery[9], was added and the mixture incubated for 60 min at 30°. Subsequently 30 µg/ml DNase solution was added at 1:30 and the culture was transferred to a bath at 37° for 100 min. At this time samples were withdrawn and diluted into two series of tubes containing maltose medium or glucose medium plus streptomycin at 150 µg/ml, respectively. The dilution tubes contained anti-pneumococcus antibody in appropriate dilution so that after 16 h incubation at 37° each transformed cell would give rise to a visible colony in the tubes[10].

In those experiments in which the DNA was heated, the heating was accomplished by placing a solution of DNA in 0.15 *M* NaCl and 0.025 *M* potassium phosphate at pH 7.0 into a water bath either at 80° or 82° for various lengths of time up to 3 h. The conditions of heating were suggested by the work of Dr. Muriel Roger of this laboratory. The solution was then cooled in an ice bath and used in the usual manner.

EXPERIMENTAL RESULTS

Enzyme deficiency of mutant strains

In contrast to lysates of the wild-type, those from mutant cells were unable to convert maltose to glucose, as is shown in Fig. 1. That the lack of activity was not

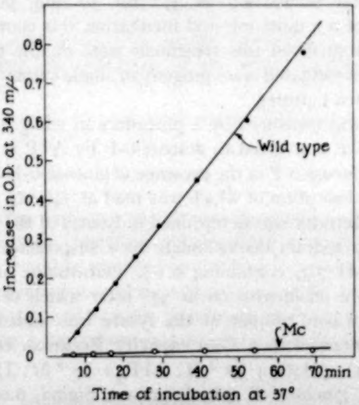

Fig. 1. Maltase activity of wild-type and mutant strains. Incubation mixture contained deoxycholate lysate of 10^7 cells, glutathione, maltose, ATP, $MgSO_4$, hexokinase, TPN and glucose-6-P dehydrogenase in Tris buffer, pH 7.2. Increase in O.D. due to reduction of TPN measures glucose liberated.

due to the presence of inhibitory material in the mutants was demonstrated by mixing lysates of wild-type and mutant cells. In all cases the activity of the mixture corresponded to the activity of the wild-type extract alone. It was possible, however, to show that after transformation of mutant cells by wild-type DNA, maltase activity appeared in the culture.

Chromatographic analyses of the products of pneumococcal maltase activity reveal the enzyme to be a transglycosylase catalysing the reaction:

$$\text{maltose} + (\text{glucose})_n \longrightarrow \text{glucose} + (\text{glucose})_{n+1}$$

Fig. 2 shows a chromatogram of the products of the enzyme action on maltose. It is seen that, initially, essentially only maltose is present, while on incubation both glucose and less rapidly migrating substances corresponding to polymers of glucose appear.

Several properties of the enzyme have been studied. Sulfhydryl compounds, of which glutathione seemed the most effective, must be added to the lysates, otherwise the activity disappears rapidly. Even with the addition of sulfhydryl compounds, however, the activity is gradually lost at 37°. After 6 h at this temperature only 60 % of the original activity remained. At lower temperatures the enzyme was more stable, although the reaction proceeded more slowly. For an incubation time of 100 min at 37° the maximal amount of glucose was formed at pH 7.0. At higher or lower pH the enzyme activity was both slower and more unstable.

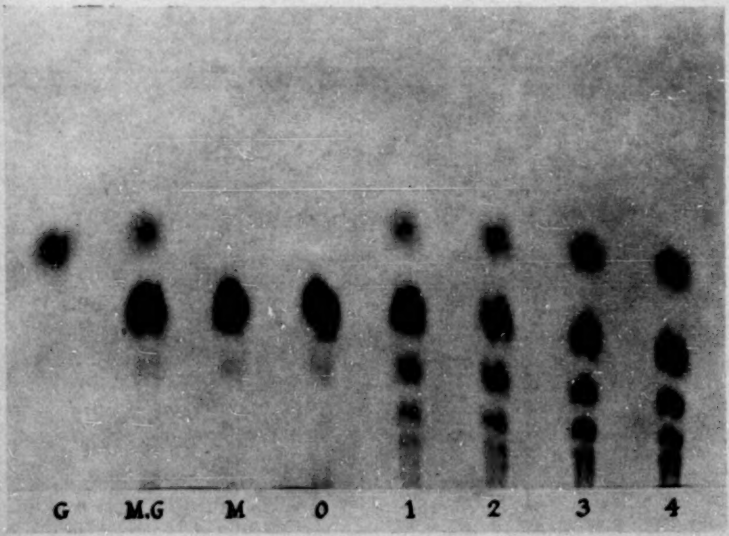

Fig. 2. Pneumococcal enzyme action on maltose. Chromatogram shows: reference spots for glucose (G), maltose (M), and the resolution of a mixture of the two (M, G); and sugars present in a mixture containing a lysate of wild-type cells, glutathione, NaF, and maltose incubated for 0, 1, 2, 3, and 4 h at 37°.

Transformation of the mutant strains with wild-type DNA

A genetic analysis of the mutants was undertaken by transforming mutant cells with DNA from the wild-type and also from other mutant strains. In all cases the same streptomycin-resistance marker was present in the DNA of the donor strain. The frequencies of transformation to maltose utilization for different mutant strains could be compared by using the frequency of transformation of the streptomycin marker as a standard of reference. This provided a measurement of the frequency of transformation to maltose utilization independent of inevitable variations in transformation efficiency occasioned by the use of many different recipient cultures frozen at somewhat different stages of growth and treated with different DNA preparations. Measured in this way the frequency was not dependent on DNA concentration.

With wild-type DNA as donor all of the mutant strains could be transformed to maltose utilization, but the ratio of maltose factor transformation to streptomycin factor transformation was different for the different mutant strains (see Table I). Characteristic frequencies are seen to vary over a fifteen-fold range.

TABLE I

FREQUENCY OF TRANSFORMATION OF MUTANT STRAINS TO MALTOSE UTILIZATION BY WILD-TYPE DNA

Values given are mean values of the ratios between yields of maltose-positive and streptomycin-resistant cells obtained in from 7 to 12 experiments.

Recipient strain	*Frequency* mal^+/str^+	*Standard deviation*
Mc	1.27	0.18
Md	0.61	0.10
Me	0.13	0.03
Mf	0.99	0.28
Mg	1.90	0.20
Mh	0.80	0.33
Mi	0.46	0.05
Mj	0.41	0.22

Recombination of genetic material of the mutant strains

When DNA from a mutant strain containing the streptomycin-resistance marker was used to transform other mutants, in certain cases the interaction gave rise to maltose-utilizing cells. A comparison of the frequency of transformation to maltose utilization by DNA from mutant and wild-type strains was made as shown in Table II for a typical experiment. As seen in the Table, the yield of maltose-positive cells is compared to the streptomycin-resistant transformants to obtain the frequency of maltose-positive transformation.

The appearance of maltose-positive cells in these crosses is taken to indicate a recombination of the genetic material corresponding to the deficiencies of the two strains. A measure of the tendency for the markers involved to recombine is obtained by comparing the frequency of such recombinant cells with the frequency of positive transformants given by the host strain with wild-type DNA. The latter frequency is taken to represent the characteristic tendency of the particular genetic deficiency of the host to be replaced by donor DNA, thereby serving as a measure of the total

TABLE II

METHOD OF CALCULATING RECOMBINATION INDEX

Ratios of maltose-positive and streptomycin-resistant cells transformed in parallel cultures by wild-type and mutant DNA's are compared to measure the tendency for recombination to occur between mutants to give positive cells.

Recipient strain	*DNA donor strain*	*Yield* mal^+	*Yield* str^+	*Ratio* mal^+/str^+	*Recombination index (ratio to wild-type)*
Mf	Wild-S	14,200	17,000	0.84	—
	MfS	< 30	7,000	< 0.005	0
	MgS	4,100	31,000	0.13	0.15
Mg	Wild-S	38,000	19,000	2.0	—
	MfS	3,700	15,300	0.24	0.12
	MgS	< 30	27,000	< 0.002	0

TABLE III

RECOMBINATION OF GENETIC MATERIAL OF MUTANT STRAINS

Summary of the recombination index found for crosses between the different mutants. Recombination at a rate of less than 0.1 % would not have been detected.

Relative frequency (in per cent) of transformation to maltose utilization by DNA from mutant and wild-type strains

Recipient strain	*DNA donor strain*							
	McS	*MdS*	*MeS*	*MfS*	*MgS*	*MhS*	*MiS*	*MjS*
Mc	—	27	0	30	15	10	0	27
Md	12	—	0	0	0	9	0	0
Me	0	0	—	0	0	0	0	0
Mf	33	0	0	—	12	15	33	5
Mg	29	0	0	13	—	9	25	5
Mh	22	17	0	21	14	—	0	15
Mi	0	0	0	10	4	0	—	9
Mj	30	0	0	5	5	11	20	—

opportunities for recombination for this marker. Recombination to give maltose-positive cells would occur only when deficient material from the donor did not at the same time replace intact parts of the host genome. Accordingly, an index of the recombination tendency is calculated by dividing the frequency of maltose-positive transformation found with mutant DNA by that found with wild-type DNA. Table III summarizes the results of crosses between all of the strains.

Effect of heating the DNA on its activity in transforming the different mutant strains

In an attempt to learn more about the physico-chemical nature of the genetic material, DNA from a maltose-utilizing, streptomycin-resistant strain was subjected to heating in order to see how its activity in transforming each of the negative strains was affected. Using the yield of transformants as a measure of the activity of the DNA, the activity of the heated DNA was compared to that of the unheated DNA in transforming each marker. Fig. 3 shows the loss of activity on heating in the transformation of strains Mc, Me, and Mi to maltose utilization and streptomycin resistance.

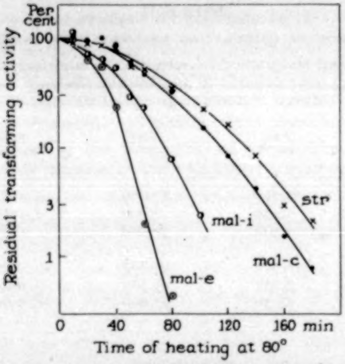

Fig. 3. Loss of transforming activity of DNA on heating at 80°. DNA solution in 0.15 *M* NaCl buffered at pH 7.0 with 0.025 *M* phosphate heated as indicated and then used to transform streptomycin-sensitive mutant strains Mc, Me, and Mi to maltose utilization and streptomycin resistance. Residual activity is determined by comparing the yield of transformants with that found with unheated DNA for the indicated markers.

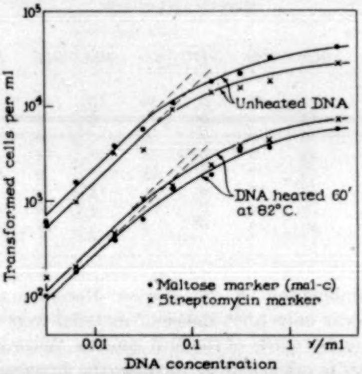

Fig. 4. Effect of heating on activity of DNA at different concentrations. Activity of unheated and heated DNA in transforming markers mal-c and str was compared at DNA concentrations in the transformation mixture falling in both the proportional and saturating regions of the concentration curve.

Comparison of heated and unheated DNA at different concentrations in the transformation mixture reveals that with heated DNA the yield of transformants is reduced to the same extent in both the proportional and plateau regions of the concentration curve. This is shown in Fig. 4 for transformation of the mal-c and str markers.

When the residual activity for maltose character transformation is compared in a logarithmic plot with the residual activity for streptomycin transformation, a linear representation of inactivation is obtained. This is shown in Figs. 5, 6, and 7 for several

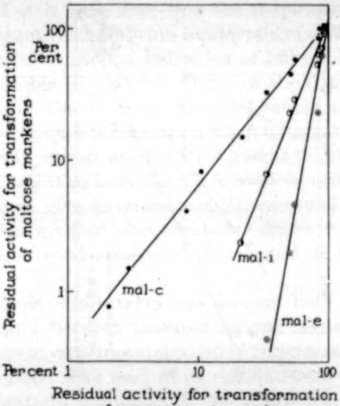

Fig. 5. Inactivation of maltose markers corresponding to mal-c, mal-i, mal-e on heating at 80°. Logarithmic decay in ability of DNA heated at 80° to transform strains Mc, Me, and Mi to maltose utilization compared to decay of ability to transform these strains to streptomycin resistance.

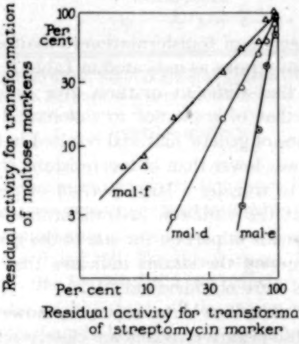

Fig. 6. Inactivation of markers corresponding to mal-f, mal-d, and mal-e on heating at 82°.

series of DNA samples heated for various lengths of time. It is observed that the ability to transform the different mutant strains to maltose utilization is lost more rapidly in certain cases than in others. For five of the strains, Mc, Mf, Mg, Mh, and Mj, the DNA is inactivated at about the same rate, while for Md and Mi the activity falls off more rapidly, and for Me, still more rapidly.

Four pyrimidine-requiring mutants of *pneumococcus* have, also, been obtained using the penicillin-selection technique. Two of the four seem to be linked. Differences in frequency of transformation and in sensitivity of the markers to heating are also observed with these mutants.

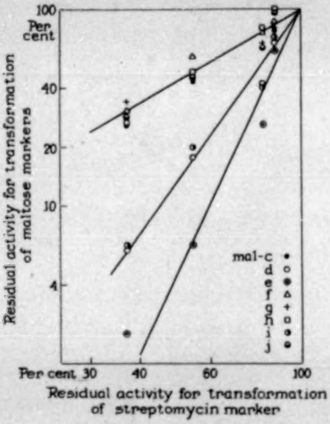

Fig. 7. Inactivation of maltose markers on heating at 80°. Results of an experiment in which loss of activity of the heated DNA was examined with respect to transformation of each of the eight maltose markers.

DISCUSSION

Different characteristic frequencies of transformation to maltose utilization were exhibited by the different mutant strains as indicated in Table I. In general it has been observed in this laboratory that different markers give different yields of transformants. In one such case, that of resistance to sulfonamides, it was found that transfer of two adjacent regions of genetic material resulted in highly-resistant transformants, the yield of which was lower than of less-resistant transformants, thereby, indicating a lesser tendency to transfer a large portion of genetic material than a small one[4]. It is possible that the variation in transformation frequency with the maltose-negative mutants depends in part on the size of the genetic regions involved. At any rate, this variation among the strains indicates that the mutations which occurred in the different cases were not identical.

An analysis of the recombination data indicates, however, that the genetic deficiencies of the eight maltose-negative strains are closely related. From Table III it is seen that some pairs of mutants fail to reveal any recombinants. This behavior, always reciprocal, is taken to indicate that the pair have mutated regions in common, that is they possess, in part, the same deficiencies. So, a mutant which fails to recombine with two others, which do recombine with each other (and therefore involve separate regions), is assumed to embrace at least portions of these two mutated regions. Similar conclusions have been drawn with regard to genetic regions in bacteriophage[11] and in bacteria[12]. Thus, the mutations observed seem to have affected regions of different extent. For instance, the Md mutation seems to affect a larger region which contains the regions affected in mutants Mf, Mg, and Mj and part of the region affected in Mi. The pattern of overlapping relationships which emerges links the regions corresponding to the eight mutations.

From Table III it is seen, also, that the frequency of maltose-positive transformation with a mutant DNA is always considerably lower than with wild-type DNA. This result is taken to be another indication of linkage between the regions in the maltase locus responsible for the deficiencies in the donor and recipient cells. The maltase deficiencies in the different mutants behave genetically as non-identical alleles[11-13]. Thus, the frequencies are lower when a "negative" mutated region linked to a "positive" (wild-type) region in the DNA lessens the probability that the entrance of positive material will transform the cell to maltose utilization. The recombination index should, therefore, give an estimate of the extent of linkage or genetic distance between regions of the maltase locus affected in different mutations. Estimates from reciprocal crosses give good agreement in the case of the small, non-overlapping regions.

The above analysis, which takes into account both the pattern of overlapping and distance estimates between mutated regions, leads to a visualization of the maltase locus as a linear structure composed of regions as shown in Fig. 8. Inasmuch as solutions of free DNA were used in its elucidation, it may be expected that this genetic map represents in some fashion the actual features of the intramolecular DNA structure.

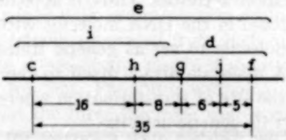

Fig. 8. Genetic map of maltase locus. Figures given as genetic distance are frequencies (in per cent) of recombination in segments between markers. Values for the reciprocal crosses between a given pair are averaged with determinations from the difference of their recombination rates with outside markers.

Genetic studies by means of transformation, as well as implicating DNA directly, provide an opportunity for exploring the relationship between the genetic map and DNA structure by manipulating the isolated genetic material experimentally before its reintroduction into a living cell. For this purpose, DNA from wild-type cells was heated. The ability of the heated DNA to transform a particular mutant would measure damage to the positive form of the region inferred to correspond to that mutant marker. It was found that the heat-sensitivity of the different regions is correlated with the structure deduced from recombination data. Just those maltose character regions which contain others and therefore appear to be larger are those which are most sensitive to heat (see Figs. 3–7). The loss of activity of those factors of the maltose locus which involve small regions, such as the regions corresponding to mutants Mc, Mf, Mg, Mh, Mj, parallels the loss of activity of the streptomycin factor. Greater sensitivity is exhibited by the factors corresponding to Md and Mi, while that corresponding to Me, which presumably is the largest region, loses its activity most rapidly on heating.

When tested at concentrations where activity is proportional to concentration, the heated sample shows lower specific activity (Fig. 4). This would result, simply, from destruction of marker material. On the other hand, at saturating concentrations, the level of transformation is determined by the ratio of marker DNA to total DNA[14]. The fact that the plateau level for the heated DNA is reduced to the same extent as the specific activity leads to the conclusion that heating destroyed the marker activity without altering the competitive activity. It appears, therefore, that under these conditions of heating, genetic activity of a DNA molecule can be altered without impairing its ability to compete with intact molecules for attachment to the cell (see LERMAN AND TOLMACH[15]).

Furthermore, if the genetic conclusions are valid, that is, if certain regions of the maltase locus are contained within others, the effect of heating the DNA must have been to produce localized injuries in the molecule. Visualizing the maltase locus as being part of a DNA molecule, and the mal-i region as a fairly large portion of that locus which in turn contains the mal-c region, injuries to the molecule in the mal-i region but not within the mal-c region would destroy the ability to transform Mi but not the ability to transform Mc. Inasmuch as a larger region would contain more sites subject to injury, the ability to transform Mi would be lost more rapidly than activity toward Mc. This, in fact, is what is observed. The alternative possibility that the DNA of the maltase locus breaks up into large and small active particles having varying sensitivity to heat seems excluded by the regularity of the decay rate for all of the small marker activities. Thus, it appears that heat can cause local destruction of genetic material in the DNA molecule without destroying the ability of other portions of the molecule to act as genetic transforming agents. Not only, then, can an injured DNA molecule attach itself to the surface of the cell, but it can also make its way to the site of transformation where an intact region adjacent to the injury can still exert its genetic activity.

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MACROMOLECULAR PARTICLES ASSOCIATED WITH ALFALFA MOSAIC VIRUS*

J. B. BANCROFT

Department of Botany and Plant Pathology, Purdue University, Lafayette, Ind. (U.S.A.)

AND

PAUL KAESBERG

Department of Biochemistry, University of Wisconsin, Madison, Wisc. (U.S.A.)

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SUMMARY

AMV was purified by differential centrifugation and *n*-butanol chloroform-water extractions at 6°. Purified preparations exhibited three closely-spaced schlieren peaks in the analytical ultracentrifuge with sedimentation coefficients of 73, 89 and 99 S. The three fractions were partially separated from each other by the use of sucrose density-gradient techniques. Local-lesion assays demonstrated that infectivity was associated with the 99-S fraction. Precipitin endpoint, cross-absorption and gel-diffusion tests indicated that the three fractions were serologically similar. Electron micrographs showed that the particles were rod-like with diameters of 200 Å and lengths 550 Å or less. The 73-S fraction contained a greater proportion of particles shorter than 550 Å than did the 99-S fraction. Approximately equal amounts of nucleic acid were associated with each of the three fractions. Attempts to show that the nucleic acid associated with the 73-S fraction was contributed by host organelles failed.

INTRODUCTION

It has been reported that at least three kinds of macromolecular particles are produced by plants as a result of their infection with alfalfa mosaic virus (AMV), and that one of these particles is the infectious entity[1]. It is the purpose of this paper to describe purification procedures and some of the biological and biophysical characteristics of these macromolecules. The production of two or more virus-like particles by virus-infected cells has been reported for turnip yellow mosaic virus[2] (TYMV), squash mosaic virus[3] (SMV), wild cucumber mosaic virus[4] (WCMV), and the bacterial virus[5] ø × 174.

MATERIALS AND METHODS

The AMV isolated[6] (AC-106) is the same as that described previously[1]. In the course of this work, three separate single-lesion subisolates were used at different times as

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checks on the biological purity of the original single-lesion isolate. Serological cross-reaction tests with eight other plant viruses[7] indicated that AMV is a distinct serological entity.

Nicotiana tabacum L. Connecticut Havana 423 which is homozygous for the tobacco mosaic virus local lesion gene from *N. glutinosa* L. was used throughout as a systemic host. The local-lesion host used was bean (*Phaseolus vulgaris* L. var Bountiful). Time-course assays made by local-lesion assays of crude sap and optical-density measurements of purified virus at 5 day intervals from 5 to 30 days after inoculation in summer and winter showed that for high virus yields, it is necessary to harvest about 10 days after inoculation in the summer (approx. 30°) and between 15 and 20 days after inoculation in the winter (approx. 20°). The virus was extracted from freshly harvested material by the methods described by Ross[8,9]. Various methods involving freezing[8], pH adjustment, *n*-amyl alcohol and chloroform–water extraction[10] and *n*-butanol and chloroform–water emulsion extraction[11] were tested in conjunction with cycles of differential centrifugation in attempts to purify the virus. The schedule which gave highly infectious, serologically specific* preparations with the least amount of centrifugation was a simple adaption of STEERE's *n*-butanol chloroform–water emulsion technique[11]. In this method, the extracted sap was clarified for 10 min at 3,400 rev./min in an Adam's safeguard centrifuge at 6°. The supernatants were transferred directly to the No. 30 rotor tubes of a Spinco Model L and were concentrated by centrifuging for 0.5 h at 29,000 rev./min. The resuspended material was clarified by centrifuging for 15 min at 15,000 rev./min. One part of *n*-butanol and one part of chloroform were added to one part of the supernatant with stirring for 15 min. The emulsion was broken by centrifuging for 15 min at 3,400 rev./min and the cloudy supernatants were allowed to stand overnight at 6°. The following morning the material was clarified by centrifuging for 10 min at 10,000 rev./min in the number 40 rotor and the resulting supernatants were concentrated by centrifugation at 39,000 rev./min for 1.5 h. The transparent pellets were resuspended in about 20% of the original volume, clarified, and then concentrated and clarified as before. This procedure resulted in a milky, slightly brown preparation (designated the unfractionated preparation) with a specific infectivity similar to that of preparations obtained by the method of Ross[8]. The brown color, the intensity of which increased with increasing age of host plants, could not be removed by dialysis against water or phosphate buffer. Separation of the virus into three components, as will be described below, showed that the coloring was associated with all three components. However, if ascorbic acid was used as described by STEERE[12], the brown color did not develop or developed to a very slight degree.

RESULTS

Analytical ultracentrifugation. Sedimentation experiments in a Spinco analytical ultracentrifuge revealed 3 schlieren peaks (Fig. 1) with sedimentation coefficients (S_{20}) from left to right of 73, 89 and 99 S. The buffer was 0.10 ionic strength of which 0.08 was contributed by sodium chloride and 0.02 by sodium phosphates. It may be seen that these peaks are not completely symmetric, showing a pronounced tailing toward the meniscus. In these experiments, and in the experiments for the biophysical data to follow, the areas under the schlieren peaks were in the ratio of 43:14:43 for top, middle, and bottom ultracentrifuge components respectively. The peaks were the same in position and relative height for preparations made by each of the reported procedures. In one experiment, preparations were made from plants infected for 8, 15, and 22 days corresponding to periods of early infection, maximum infection and partial recovery; the schlieren positions and ratios remained unchanged. It is not known if the sedimentation data are invariant for all strains of the virus, but the schlieren patterns obtained with strains AC-109 and AC-106 showed no differences[6].

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* Tested by ring-precipitin and gel-diffusion methods using antisera prepared by both intravenous-saline and intramuscular-adjuvant (FREUND, incomplete) injection techniques against preparations from diseased and healthy plants.

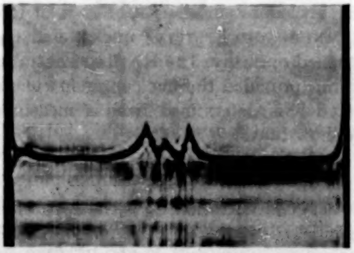

Fig. 1. Ultracentrifugation pattern of an unfractionated preparation of AMV showing top, middle and bottom components. Sedimentation is from left to right.

Separation of the three ultracentrifuge components. Our experience with corresponding macromolecular components resulting from infection with squash mosaic virus[13] suggested that chemical separation or selective destruction of the components would be difficult. Nevertheless several standard techniques, such as ammonium sulfate fractionation, calcium phosphate adsorption, and pH adjustment, were attempted but without success. All three components were fragmented into lower mol. wt. units, at pH's below 4.4 and above 9.

Density-gradient separations were made as follows: Columns of 10, 20, 30, and 40% sucrose in 0.01 M, pH 7.0 phosphate buffer in 4, 7, 7, and 7 ml amounts, respectively, were prepared with thin-stemmed funnels and allowed to stand overnight in the cold. Alternatively, 10 to 40% gradients were prepared using a gradient-making apparatus[4]. 2 ml aliquots of the unfractionated preparation at a concentration from 3.0 to 3.5 mg/ml were centrifuged in a S.W.-25.1 Spinco swinging-bucket rotor for 2 to 2.75 h at 24,000 rev./min. These conditions resulted in a reasonably good resolution of three opalescent bands corresponding to the three ultracentrifuge components. The tubes were punctured at suitable levels with hypodermic needles and the layers were removed. The fractions thus obtained were then sedimented at high speed, brought up in buffer, and subjected to biophysical, serological and infectivity tests.

Three fractions containing 90% top component, 90% bottom component and 50% middle component, respectively, could be achieved in a single cycle of density-gradient sedimentation. In the discussion to follow these fractions will be designated as top fraction, bottom fraction and middle fraction. More complete separations were achieved by repeated density-gradient sedimentations on these partially separated fractions. However, the latter experiments have not been pursued at length because of the large quantities of material required and also because the infectivity of all of our preparations decreased markedly with time.

Ultraviolet absorption and the estimation of nucleic acid concentration. Each of the three fractions and also the unfractionated material, containing all three components, gave identical ultraviolet absorption curves with a minimum at 242 mμ and a maximum at 258 mμ. The ratio of the absorption at 260 mμ was 1.65.

If the specific absorption at 260 and 280 mμ for the RNA and the protein in these samples were identical with that of RNA and protein in tobacco mosaic virus[14] then this absorption ratio implies that each sample contains 3.6 times as much protein as it does RNA, *i.e.*, these nucleoproteins consist of 22 % of RNA and 78 % protein.

According to ENGLANDER AND EPSTEIN[15] nucleic acid and nucleoprotein concentrations may be determined optically. The RNA concentration is obtained from the optical density at 260 mμ provided the nucleoprotein concentration is known. The nucleoprotein concentration is determined from a measurement of the refractive index increment. They show that:

$$C_n = C_a - \frac{C_{np} - C_a}{R - 1}$$

where C_n is the nucleic acid concentration, C_a is the apparent nucleic acid concentration obtained from the optical density. C_{np} is the nucleoprotein concentration obtained from the refractive index increment measured in the ultracentrifuge. R is the ratio of the extinction coefficient of nucleic acid to that of protein at 260 mμ. The optical density of RNA was taken to be 25.3 for a 1 mg/ml solution. The specific refractive increment of nucleoprotein was taken to be 0.00180. R-1 was taken to be 40. The nucleic acid, nucleoprotein ratios (expressed as percents) were found to be 21, 24, and 20 % in the top, middle, and bottom fractions, respectively.

The nucleic acid concentration of the original unfractionated preparation containing all three components, was determined from a phosphorus analysis to be 21.5 %.

Serology. The serological relationship among the three components was established by using virus end-point, cross-adsorption and gel-diffusion methods. Experiments in which equal amounts of the three fractions (as determined by ultraviolet absorption) were reacted against antiserum, prepared by intravenous injection of the unfractionated preparation, showed that the three fractions had the same end-point and also that the sizes of the precipitates were similar for each dilution (Table I). It is possible that each component reacted with its corresponding type of antibody only. Thus antiserum was prepared against a small amount of bottom fraction by adjuvant injections and the three fractions were, once more, used in tube precipitin and ring interface tests. The results again showed that the three fractions reacted similarly (Table I) indicating a close relationship.

In a cross-absorption experiment, 2-fold dilutions of equalized top, middle, and bottom fractions were reacted in a tube precipitin test against antiserum prepared by intramuscular adjuvant injection of the unfractionated preparation. The precipitates resulting from the reactions of the three components against the 1/8 antiserum were similar in size at the various dilutions thus confirming the results obtained using antiserum prepared by intravenous injection. The supernatants from tubes showing the greatest precipitates were collected after centrifugation and tested against 2-fold

TABLE I

THE SEROLOGICAL RELATIONSHIPS AMONG THE THREE COMPONENTS OF AMV AS ESTIMATED BY END-POINT READINGS

Type of antiserum	*Test material*	*Reciprocal of virus end-point as determined by*	
		Tube precipitin test*	*Ring interface** precipitin test*
Unfractionated preparation (1/32)	Top fraction	16	128
	Middle fraction	16	128
	Bottom fraction	16	128
Bottom fraction (1/16)	Top fraction	64	512
	Middle fraction	64	512
	Bottom fraction	64	512

* Final reading after being left overnight at 4°.

** Final reading after 2 h at room temperature.

dilutions of the unfractionated preparation using the sensitive ring test. No ring-precipitin reactions occurred in the cross absorbed serum, but did in the control (Table II). It was permissable to use the unfractionated preparation in the ring tests because it had previously been determined in an experiment in which antiserum from the unfractionated preparation had been cross absorbed against top and bottom fractions, that equalized preparations of the unfractionated preparation, top, and bottom fractions, did not react against the cross-absorbed antiserum.

In a gel-diffusion test, top, middle and bottom fractions were allowed to react in agar in a petri plate against antiserum. The precipitin lines between all pits coalesced.

From these tests, and assuming that the top and middle components as well as the bottom component are antigenic, it is concluded that the components are serologically similar.

Infectivity. The relative infectivities of the three fractions were ascertained by local-lesion assays on bean. Since the separated components gave similar ultraviolet absorption curves, optical density was employed as a measure of concentration and this was checked serologically. Because only small amounts of the separated fractions were available the use of weight as a basis for equalizing the concentration was impracticable. The results are summarized in Table III. Each of the five experiments

TABLE II

THE SEROLOGICAL RELATIONSHIPS AMONG THE THREE COMPONENTS OF AMV AS DETERMINED BY CROSS-ABSORPTION

Component used to cross-absorb antiserum to unfractionated preparation	*Dilution of unfractionated preparation reacted against cross-absorbed antiserum*									
	*1/1**	*1/2*	*1/4*	*1/8*	*1/16*	*1/32*	*1/64*	*1/128*	*1/256*	*1/512*
Top	—	—	—	—	—	—	—	—	—	—
Middle	—	—	—	—	—	—	—	—	—	—
Bottom	—	—	—	—	—	—	—	—	—	—
Saline	+	+	+	+	+	+	+	+	—	—

* 0.069 mg/ml initial virus concentration in ring precipitin test.

listed therein constitutes a separate unit because they were run at different times using different preparations. Since it is difficult to standardize the degree of virus inactivation and many of the testing conditions, the lesion counts from different experiments are not directly comparable. It may be seen from the result of experiment 1 that the infectivity per optical density unit and per serological unit was greatest for the bottom fraction. In Expts. 2, 3, and 4, in which the amounts of the fractions were varied it is clear that the lesion numbers changed in accord with the amount of the bottom fraction. In expt. 5, equal amounts of the three fractions were added to each other in all combinations. Since the activities of the three fractions on the set of plants were known, the expected numbers of lesions could be predicted in the combined preparations. If we let the ratio of the lesion counts for top: middle: bottom fractions equal 0:27:100 (as found in Expt. 5, Table III), then ratios of 13:63:50:42 for (top + middle):(middle + bottom):(bottom + top):(top + middle + bottom) preparations, respectively, would be expected. The actual ratios of 12:70:55:49 are in good agreement with the expected values and show that the low infectivities associated with the middle and top fractions probably are not a result of virus inhibitors but rather are a result of the intrinsic inability of the particles to cause infection.

These results show that the bottom fraction is infectious and that the middle and top fractions are less infectious or are non-infectious. It will be recalled that the top fraction contains a small admixture of middle and bottom components and that the

TABLE III

THE RELATIVE INFECTIVITIES OF THE TOP, MIDDLE AND BOTTOM FRACTIONS

Expt. No.	*Test material*	*Optical density at*		*Reciprocal of serological end-point**	*Local lesions***
		260 mμ	*280 mμ*		
1	Top fraction	0.163	0.099	16	15
	Middle	0.166	0.101	16	534
	Bottom	0.168	0.100	16	1666
2	Top	0.109	0.064	16	0
	Middle	0.350	0.208	64	267
	Bottom	1.669	0.978	512	1796
3	Top	0.709	0.438	128	63
	Middle	0.074	0.044	16	135
	Bottom	0.717	0.447	128	2309
4	Top	0.955	0.565	256	16
	Middle	0.286	0.170	32	236
	Bottom	0.062	0.037	8	179
5	Top	0.124	0.067	16	1
	Middle	0.125	0.068	16	87
	Bottom	0.124	0.067	16	324
	Top + middle	0.123	0.067	16	38
	Middle + bottom	0.122	0.067	16	228
	Bottom + top	0.125	0.070	16	179
	Top + middle + bottom	0.125	0.070	16	158
	Unfractionated preparation	0.124	0.069	16	574

* Ring test reading after 2 h at room temperature.
** Total local lesions on 8 half leaves.

middle fraction contains a considerable proportion of top and bottom components. Thus the infectivity found in the bottom fraction is accountable to the presence of infectious bottom component, and the infectivity in the top and middle fractions can be accounted for completely on the basis of contamination with the bottom component.

Electron Microscopy. Electron micrographs of the three fractions were obtained by standard procedures. For air-dried preparations each of the solutions was diluted suitably with water and sprayed with a Vaponefrin[*] nebulizer onto collodion films supported on 200-mesh Athene specimen grids. For frozen-dried specimens, the grids were mounted on copper blocks and cooled to a temperature of —70°. After spraying, vacuum sublimation was carried out at a temperature of —40° following essentially the procedure described by WILLIAMS[16].

As has been described briefly[1] the electron micrographs of the fractions were virtually indistinguishable. They showed short rods, very uniform in width, about 240 Å, but inhomogeneous in length. It is apparent from the dimensions of the shadows cast by the particles that their thickness, in air-dried as well as in frozen-dried preparations is approximately equal to their width. Thus they are sufficiently sturdy to resist flattening. Presumably, a more reliable measure of particle width is the axis-to-axis separation of contiguous particles. This dimension is about 200 Å. Fig. 2 shows the length distribution among 100, aimlessly chosen, particles from each of the three fractions. It is apparent from Fig. 2 that there are only a few particles longer than about 550 Å, that a majority of the particles in the bottom fraction are about 550 Å long and that the top fraction has a greater proportion of particles shorter than 550 Å. Electron micrographs of these fractions are shown in Fig. 3. The length distribution for the middle fraction is similar to that of the top fraction but because of the large admixture of top and bottom component particles in this fraction the validity of the length distribution shown for it is doubtful.

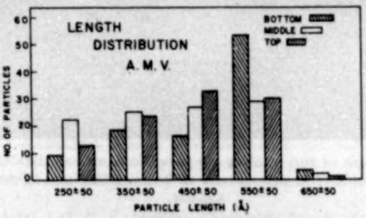

Fig. 2. Length distribution among 100 particles, each, of bottom fraction, middle fraction, and top fraction.

DISCUSSION

The results suggest that AMV is a short rodlet with a sedimentation rate of about 99 S (bottom component) rather than a spherical, 70-S particle as had been believed previously[17]. They also disclose the existence of two other macromolecular particles, with sedimentation rates of 73 and 88 S, apparently very similar in structural and chemical properties to AMV. It now seems likely that Ross's original isolate or purification

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[*] Vaponefrin Co., Upper Darby, Pa. (U.S.A.).

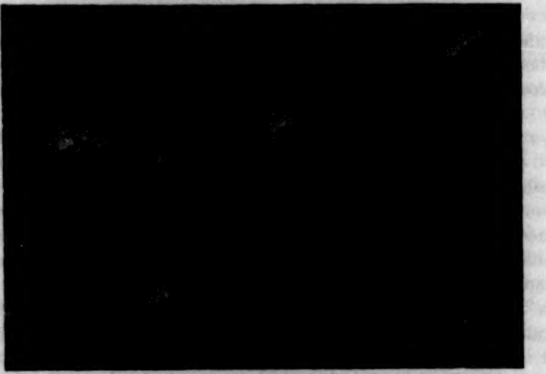

Fig. 3a. Electron micrograph of bottom fraction taken with a Siemens Elmiskop I. Shadowed with uranium. Magnified 140,000 ×.

Fig. 3b. Electron micrograph of top fraction prepared in a manner like that of Fig. 3a, showing that top fraction is very similar in appearance to bottom fraction. Middle fraction is also very similar.

schedule resulted in a relatively large proportion of the 73-S component which would have made the slightly more rapidly sedimenting 99-S component very difficult to observe with the ultracentrifuge techniques available at that time.

While it now seems certain that AMV is to be associated with the bottom component, this component, itself, consists of a mixture of particle sizes as judged by the electron microscope pictures and by the asymmetry of the ultracentrifuge peak. By analogy with the studies on tobacco mosaic virus, we may use as a working hypothesis that the longest, and at the same time the most abundant rod, is the infectious unit and that the shorter rods are broken pieces of it. Because of its small dimensions and also because of the lability of the AMV infectivity, verification will almost certainly be even more difficult than were the corresponding experiments with TMV[18].

At present, we are proceeding indirectly on this problem by attempting depolymerization and repolymerization experiments designed to determine whether the AMV protein has a spiral subunit structure similar to TMV.

The existence is well documented of non-infectious macromolecules similar in their protein portions to their corresponding viruses[2,4,11]. In each case the difference in sedimentation rate from that of the corresponding virus was accountable to its lesser content (or complete absence) of RNA. In the present instance with AMV, the optical data suggest that the nucleic acid content of the top and bottom components is similar and hence does not account for the difference in sedimentation rate. Furthermore, the length distributions for top and bottom components are not sufficiently different to account for a 25 % difference in sedimentation rate. The possibility that the 73-S peak represents a mixture of a virus-like macromolecule and another component with a large RNA content is not ruled out, although such particles were not detected in control preparations.

The isolation of the middle component was not clean enough to merit further discussion.

It is evident that the limiting factor in biophysical studies of AMV is the separation of the components in sufficient quantity and purity for meaningful experiments. The lability of the infectivity is a contributing consideration. Nevertheless, the novel structure of these macromolecules suggests that serious efforts to overcome these experimental limitations are warranted.

ACKNOWLEDGEMENTS

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Short Communications

The reaction of porcine and ovine ribonucleases with antibody to bovine ribonuclease

The amino acid sequences of bovine and ovine pancreatic RNase differ at three residues, although both enzymes have a similar specific activity[1,2]. Studies on the structure of the porcine enzyme have shown no obvious differences in sequence from that of bovine RNase[3]. However, the methods so far employed would not detect minor variations in the exact sequence or in the pairing of half-cystine residues in disulfide bonds and the observations on the porcine enzyme reported here are, therefore, preliminary. The combination of antibody molecules with several sites of RNase gives information concerning certain structures that do not appear to be involved in the enzyme activity[4]. Immunologic studies were undertaken to determine whether substitution of amino acids at these points influences the reaction of RNase with its antiserum and to help elucidate the chemical nature of these antigenic sites[5].

Antisera were prepared in rabbits by the intravenous injection of increasing doses of alum-precipitated bovine RNase A three times a week until 13 doses totaling 40 mg of protein had been given. The rabbits were bled a week after the last injection and the serum heated to 56° for 30 min before use. The second-course serum was prepared by giving a similar course of immunization 9 weeks after bleeding. The initial dose was injected intraperitoneally. To make precipitin analyses, dilutions containing increasing amounts of RNase were added to 0.5 ml of antiserum. The mixtures were stored at 0° for 3 days and washed in the usual manner[6]. The amount of precipitate was determined by nitrogen analysis[7]. Ovine RNase did not precipitate as much first-course antibody as did bovine RNase (Fig. 1). When either ovine or bovine RNase solution was placed

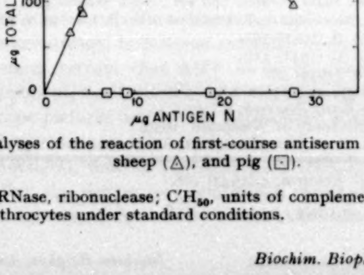

Fig. 1. Precipitin analyses of the reaction of first-course antiserum with RNase from cow (⊙), sheep (△), and pig (⊡).

Abbreviations: RNase, ribonuclease; $C'H_{50}$, units of complement, the amount giving 50% lysis of sensitized erythrocytes under standard conditions.

over a 1:1 mixture of 1 % agar and antibody, each gave a single strong line of precipitation that moved gradually down the tube. A solution of porcine RNase containing 16.4 μg N in 0.1 ml placed in a similar tube gave no precipitate. The porcine RNase used was, however, enzymically active.

The immunologic reactivity of the RNases from the cow, sheep, and pig was also tested by complement fixation[8]. Aliquots with increasing amounts of antigen were added to a mixture of diluted antiserum and a dilution of guinea-pig serum containing one hundred 50 % hemolytic units of complement. After storage for 18 h at 0°, the amount of complement left was determined and subtracted from the average of the amounts in control tubes which lacked antigen or antibody, or both. The extent of reaction of antibody with antigen is related to the amount of complement destroyed or fixed. The reactivity of the antigens was compared from the maximal amount of complement fixed by each. When first-course antibody was used, ovine RNase reacted only partially and porcine RNase was inert even at high concentrations (Table I). Ovine RNase reacted well and porcine RNase partially with second-course antiserum (Fig. 2). All three antigens gave precipitates with second-course serum in the agar-gel system. Complement-fixation results were again in agreement with precipitin analyses (Table I). In all, only 6 different antisera were used but experience with other partially reactive RNase derivatives suggests that all sera with a given combining ratio of bovine RNase to antibody will behave similarly. The ratio of antibody to antigen was increased when second-course sera were used, probably as the result of antibodies to an additional site on RNase. This would account for the increased reactivity of ovine and porcine RNase with these antisera. The results might be due, however, to improved fit of antigen and antibody and/or increased synthesis of a particular natural globulin[9].

TABLE I

COMPLEMENT-FIXATION RESULTS WITH RNASE FROM VARIOUS SPECIES

The sera were diluted so that 1.0 ± 0.1 μg of antibody N was used for each point. One other first-course serum tested with the 3 antigens and one other second-course serum tested with ovine and bovine RNase gave similar results.

μg Antigen N	*RNase*		
	Bovine	*Ovine*	*Porcine*
	C'H$_{50}$ fixed with first-course serum		
0.025	25	26	3
0.05	43	45	0
0.10	71	53	1
0.20	66	45	4
0.40	44	28	2
0.80	32	19	3
1.60	*	*	2
3.20	*	*	1
	C'H$_{50}$ fixed with second-course serum		
0.009	32	29	15
0.019	41	39	16
0.038	63	60	22
0.075	78	79	27
0.15	55	67	32
0.30	*	25	22

* Not determined.

When large amounts of antigen are present, less complement is fixed by a given amount of antibody. Even modified antigens, which do not precipitate with antibody or fix complement, often decrease the amount of complement fixed by a given antigen–antibody mixture[4]. Increasing amounts of porcine RNase added to a mixture of complement, bovine RNase, and first-course antiserum decreased the amount of complement fixed, although a given amount was less effective than a similar quantity of bovine RNase.

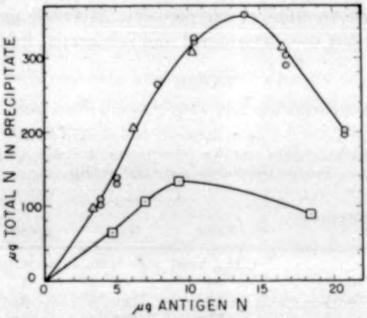

Fig. 2. Precipitin analyses of the reaction of second-course antiserum with RNase from cow (⊙), sheep (△), and pig (⊡).

Immunologic studies of Edman degradation products of guanidinated RNase indicate that residues 1 and 2 are necessary for immune reactivity[4] and it is possible that the threonine residue in position 3 or the lysine residue at position 37 is also a site of combination of antibody with RNase. However, it is equally possible that these residues, especially the lysine at residue 37, merely hold the parts of an antigenic site in appropriate position. In this case substitution of a glutamic acid residue in position 37 might alter the spacing between as yet unknown portions of the site and cause a decrease in reactivity.

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Division of Laboratories and Research, New York State Department of Health, Albany and National Heart Institute, National Institutes of Health, Bethesda, Md. (U.S.A.)

Ray K. Brown
Barbara C. Tacey
Christian B. Anfinsen, Jr.

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Inhibition of amylases by rooster antisera to hog pancreatic amylase

Antisera containing antibodies which inhibited the enzyme activities of certain amylases[1] were produced by the injection of crystalline hog pancreatic amylase into rabbits. By use of these antisera, it was demonstrated that hog liver amylase was different from hog pancreatic, salivary and serum amylases. These latter amylases were strongly inhibited by the antisera whereas the liver amylase was not significantly inhibited. This result was not the expected one since in many cases the enzymes of a given type produced by different organs of the same species are identical, *e.g.*, human salivary and pancreatic amylases. For this reason, we wished to confirm the initial findings.

HENION AND SUTHERLAND[2], GREGORY AND WROBLEWSKI[3], among others, have shown that enzyme-inhibiting antibodies may successfully be produced in roosters. To see whether antibodies to amylase could be formed in the rooster, 4 adult roosters were first bled weekly for 6 weeks to obtain normal sera and then injected intradermally with crystalline hog pancreatic amylase (Worthington Biochemical Corp.) emulsified in FREUND's[4,5] adjuvants, omitting the heat-killed bacteria. The injections were given as follows: 25 mg each, divided between 5 or 6 sites, were injected into the backs of the roosters. Two weeks later blood was drawn and the enzyme-inhibiting capacity of the antisera tested. At this time, it was less than 10 % in 2 and 40 % in the other 2. Second injections of 10 mg each of hog pancreatic amylase in aqueous solutions were given just after the drawing of blood. Two weeks later blood was again drawn and antisera obtained.

In 2 of the 4 roosters, the antisera obtained inhibited the original antigen, hog pancreatic amylase only 15 % and so were discarded. However, antisera from the other 2 would inhibit hog pancreatic amylase 67 % and 75 % respectively at 1:20 dilutions under the conditions originally described[1]. These two roosters were then bled to death to obtain all the antisera possible. Further immunization of the roosters was not continued in order to avoid the possibility that the enzyme-inhibiting antibody titers might fall[3] and that the specificity of the antibodies formed might become less with increasing numbers of injections.

As with rabbit antisera, equal volumes of the amylase solution containing about 1–2 amylase units/ml and a 1:20 dilution of the antiserum in 0.9 % NaCl were mixed, allowed to stand for 40 min, a 1-ml aliquot removed and the amylase activity determined by the amyloclastic method of VAN LOON *et al.*[6]. The amylase-inhibiting properties of these antisera on other amylases are shown in Table I. Appropriate controls were run to determine that there was no inhibition of amylases by normal rooster sera, and to determine the activities of the pancreatic amylases in the presence of rooster serum proteins. It was previously determined[1] that added protein will enhance the activity of pancreatic or salivary amylases at high dilutions.

Antibodies to hog pancreatic amylase were also produced in the rat by the injection of 10 mg of the amylase emulsified as previously described followed by a second injection of 5 mg in aqueous solution two weeks later. Antisera collected two weeks after the second injection inhibited hog pancreatic amylase 52 and 66 % when used at 1:100 dilutions.

The results with hog amylases confirm the previous finding[1] that hog liver amylase is different from other hog amylases. The difference in the inhibitions of hog pancreatic and serum amylases indicates that at least some part of the hog serum amylase is derived from a source other than the pancreas. If the response to an antibody by two enzymes is the same, one can only say that the two enzymes may be the same. However, if the responses are markedly different, the enzymes must have some structural differences even though their catalytic properties are similar.

TABLE I

INHIBITION OF VARIOUS AMYLASES BY ANTIBODIES TO HOG PANCREATIC AMYLASE

Amylase from	*% inhibition of original amylase activity**		
	Hog	*Dog*	*Rat*
Pancreas	72	8	48
Serum	39	18	0
Liver	6	16	5

* Means of the averages of duplicate or triplicate determinations with antisera from each of the two roosters which produced significant antibody titers.

There were some cross reactions of dog amylases with the rooster antisera although the pattern was somewhat different from that seen[1] with rabbit antisera. The large difference in the inhibition of rat pancreatic amylase compared to rat liver and serum amylases indicates that the pancreatic amylase differs in some respect from the other two. This is further confirmation of the earlier conclusion[7] that rat serum amylase must be derived from some source other than the pancreas. This conclusion could not have been drawn from the effect of rabbit antisera on rat amylases and points out the advantage in these studies of using antisera prepared in more than one animal.

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Department of Biochemistry, University of Louisville, ROBERT L. MCGEACHIN
School of Medicine, Louisville, Ky. (U.S.A.) JAMES M. REYNOLDS

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A rapid method for the separation of plasmals

The lack of delicate methods for separating and identifying long-chain aldehydes is partly responsible for retarding the study of plasmalogen metabolism. Several different aldehydes are known to be released from plasmalogens[1-3] and gas chromatography may reveal many more[4]. All of the methods for separating the released plasmals either require large amounts of material or are too time consuming to be routinely applied[5,6].

The method which is reported here was developed from the procedure used by GADDIS AND ELLIS[7] in studying lower aldehydes. Micro-quantities of materials can be separated and identified chromatographically as the 2,4-dinitrophenylhydrazones very readily even in the presence of complex lipid mixtures.

In the present study, all reagents and solvents were of A.C.S. grade unless otherwise stated. The 2,4-dinitrophenylhydrazine was purified by repeated recrystallization from butanol. Ethanol was refluxed over 2,4-dinitrophenylhydrazine and distilled. Petrolatum (Snow White Brand) was used for impregnation of sheets of Whatman No. 3 filter paper. The other brands of petrolatum tried were found to contain excessive quantities of u.v.-absorbing materials. Filter-paper sheets for chromatography were impregnated by dipping in a 2% solution of petrolatum in petroleum ether (b.p., 65–110°). After air drying, the sheets were cut into strips 4 in. × 10.5 in., the long axis following the machine direction.

Known 2,4-dinitrophenylhydrazone derivatives were prepared from palmitaldehyde and stearaldehyde (K and K Laboratories) and myristaldehyde (Matheson, Coleman and Bell, Inc). These aldehydes were converted to the corresponding dimethyl acetals which were purified and reacted with 2,4-dinitrophenylhydrazine by the method of IDDLES *et al.*[8]. The products were recrystallized from methanol and ether until a constant melting derivative was obtained.

Palmitaldehyde dinitrophenylhydrazone formed bright-yellow crystals, m.p. 105–6°; absorption max. (in CCl_4), 345 mμ (ε_M, $2.19 \cdot 10^4$) (Found: N, 13.53. Calc. for $C_{22}H_{36}N_4O_4$: N, 13.32.) Stearaldehyde dinitrophenylhydrazone formed bright-yellow crystals, m.p. 106–7°; absorption max. (in CCl_4), 345 mμ (ε_M, $2.14 \cdot 10^4$) (Found: C, 65.19; H, 9.18; N, 12.46. Calc. for $C_{24}H_{39}N_4O_4$: C, 64.25; H, 8.99; N, 12.49.)

Stock solutions of these compounds prepared in CCl_4 were stored in the refrigerator for many months without any apparent decomposition. Samples of olealdehyde and linolealdehyde dinitrophenylhydrazones were generously supplied by Dr. H. MANGOLD[9].

The 2,4-dinitrophenylhydrazide of palmitic acid was synthesized by a modification of the method of INOUYE AND NODA[10].

Lipid extracts were prepared from tissues by the method of FOLCH[11], although lipid extracts prepared by other means were also suitable. An aliquot of the extract containing about 100 μg of plasmal was taken to dryness under a stream of nitrogen at 50° in a centrifuge tube and the residue was suspended in 2 ml of purified ethanol. 2 ml 2 *N* HCl and 1 ml of a saturated solution of 2,4-dinitrophenyldrazone in 2 *N* HCl were added. The mixture was allowed to react for 30 to 120 min, after which 4 ml petroleum ether (b.p., 30–60°) was added to extract the reaction product. After shaking, the lower layer containing the unreacted reagent was removed with the aid of a capillary pipette. The petroleum ether phase was shaken with 1 ml methanol and the lower methanolic phase was discarded. Recovery experiments have shown that this procedure removes the bulk of the lower dinitrophenyl hydrazones and other more polar materials with negligible losses of the long-chain derivatives. An aliquot of the petroleum ether extract was then spotted for chromatography.

The chromatographic chambers used consisted of glass cylinders 6 in. × 12 in. sealed with glass plates to which were attached clips for holding paper strips. Mercury traps were used to regulate the pressure within the chambers. The solvent used for chromatography was methanol–water (96:4). Equilibration of the paper with solvent prior to running did not appear to have any advantage. The samples were applied to the paper in streaks or spots 1/in. from the bottom of the sheet. A mixture of known dinitrophenylhydrazones was run simultaneously with the tissue samples for identification purposes. Usually from 12 to 14 μg of each derivative is required for quantitative recovery while as little as 2 to 3 μg is sufficient for qualitative identification.

The strips were immersed 0.25 in. in the solvent and chromatography was allowed to proceed for about 4 h in the ascending direction. At the end of this time the chromatographs were removed and examined under long-wavelength u.v. light. The dinitrophenyl hydrazone spots or streaks, which appeared dark against a white background, were marked with pencil.

Further treatment of the strip depended on the nature of the experiment. Qualitative identification of the aldehyde derivatives was made by comparing the positions of the unknowns with those of the reference standards. Quantitative estimation was carried out by transferring hydrazone-containing sections of the paper to a coarse micro sintered-glass filter, eluting with 2.0 ml CCl_4, and measuring the absorbancy of the eluate in a Beckman spectrophotometer at 345 mμ. The elution was complete if the solvent was recycled through the filter 10 to 15 times with a medicine dropper. A non-hydrazone containing section of the chromatograph was carried

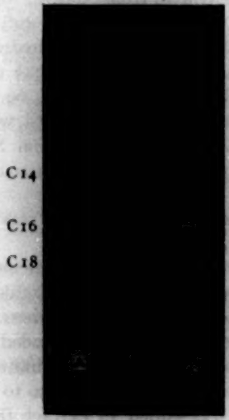

Fig. 1. Paper chromatogram of dinitrophenyl hydrazones. Reproduced as direct contact print. A, synthetic mixture of C_{18}, C_{16}, C_{14} saturated aldehyde dinitrophenylhydrazone derivatives. B, C, two samples of aldehyde dinitrophenylhydrazones from rat brain. Solvent: MeOH–H_2O (96:4, v/v).

through as a blank. In experiments where radioactive materials were used an aliquot of the eluate was counted for the determination of the specific activity.

Fig. 1 shows a typical chromatograph. This is reproduced as a direct contact print on Kodak Azo F-4 single-weight paper. The separations are more clearly seen under u.v. light than in this photograph. The R_F values averaged 0.47, 0.36, and 0.24 for the C_{14}, C_{16} and C_{18} saturated aldehydes respectively (Fig. 1, A). The dinitrophenylhydrazones of palmitaldehyde and olealdehyde, and of myristaldehyde and linoleaIdehyDe, form inseparable pairs in this system, but the latter two have not been found to occur in any of the tissues studied. A second system is being developed for the separation of the unsaturated dinitrophenylhydrazones. The dinitrophenylhydrazide of palmitic acid runs far ahead of these compounds (R_F, 0.8) as do the dinitrophenylhydrazones of the lower aldehydes.

Human blood, and rat brain, liver, heart muscle, adrenal glands and testes have been examined using this method. In all cases C_{16} and C_{18} dinitrophenylhydrazones were found although the relative proportions of these materials differed (Fig. 1, B,C are representative). In every instance, if sufficient material was run, a spot having an R_F value of 0.14 was also present. This spot was less intense than that of the C_{16} or C_{18}, but still very distinct. We believe that it represents the dinitrophenylhydrazone of a C_{20} (or higher) aldehyde, to the authors' knowledge hitherto unreported. This will be confirmed when synthetic eicosanal is prepared. No spot with an R_F near 0.47 was seen, ruling out the presence of appreciable myristaldehyde and linoleaIdehyde.

The method described above is a rapid, sensitive, and easily performed means of separating plasmals. If the volumes of solutions are proportionately reduced, it can be applied to milligram quantities of brain tissue. Other methods require much larger samples. This method is valuable in studying the incorporation of labeled precursors into the aldehydogenic group of plasmalogens, because one can accurately determine the specific activity of the individual dinitrophenylhydrazone fractions with small amounts of materials.

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Departments of Agricultural Biochemistry and Physiological Chemistry Ohio State University, Columbus, Ohio (U.S.A.)
R. W. Keenan[*]
Bernard H. Marks

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[*] National Science Foundation fellow. Submitted in partial fulfillment of requirements for the Ph.D. degree.

A colorimetric assay for 2-heptanone and other ketones

This assay is based on the formation of the 2,4-dinitrophenylhydrazone of 2-heptanone, and the subsequent formation of a colored complex with NaOH. The principle of this method was first described by DAKIN AND DUDLEY[1], and various modifications were described by other workers[2,3]. In the investigations presently being done in our laboratory, the procedure is used primarily to determine quantitatively small amounts of the methyl ketone, 2-heptanone. This ketone is produced by the conversion of caprylic acid to 2-heptanone (methyl amyl ketone) by spores of the mold *Penicillium roqueforti*.

The assay procedure requires the following: A standard containing 2.0 μmoles 2-heptanone/ml of distilled water is prepared. This standard must be freshly prepared, since the ketone is quite volatile. The hydrazine used is a 0.1 % solution of 2,4-dinitrophenylhydrazine prepared in 2 *N* HCl. 100 mg 2,4-dinitrophenylhydrazine is dissolved in 100 ml 2 *N* HCl and filtered. This reagent can be stored indefinitely if kept refrigerated. 95 % ethyl alcohol is used to solubilize the phenylhydrazone, and 6 *N* NaOH is used to form the colored complex.

The unknown sample (and/or standard) is added to 0.5 ml of the 2,4-dinitrophenylhydrazine solution in an 18 mm matched colorimeter tube, and made to a total volume of 4.5 ml with distilled water. This is immediately shaken and allowed to stand exactly 20 min. If a series of unknowns are being assayed, the samples are added to the phenylhydrazine reagent at 30-sec intervals; all subsequent operations are also timed at 30-sec intervals. After the first 20 min, 3.0 ml 95 % ethanol is added and the tube is shaken vigorously. It is again allowed to stand 20 min, after which 1.0 ml 6 *N* NaOH is added with vigorous shaking. After standing an additional 10 min, the absorbancy is read on a colorimeter (Bausch and Lomb Spectronic 20) at a wavelength of 540 mμ.

The methods described by DAKIN AND DUDLEY[1], FRIEDMAN AND HAUGEN[2], and GREENBERG AND LESTER[3] all involve extraction procedures in various solvents as a means of separating the different ketones, aldehydes, and keto acids under consideration. These extraction procedures can be applied to this assay; however, since we are working with only one ketone, their use is unnecessary. Most of the short-chain ketones, aldehydes, and keto acids are readily soluble in NaOH. Since 2-heptanone is only slightly soluble in NaOH it is necessary to dissolve it in an organic solvent. Ethyl alcohol was chosen because of its miscibility with water, which eliminates the necessity of removing the water. The color formed upon the addition of NaOH is greatly intensified in the presence of ethyl alcohol; it is also a more stable color. The influence of the alcohol on the assay is illustrated in Fig. 1.

Although the formation of the 2,4-dinitrophenylhydrazone of 2-heptanone is not quite complete within the time allotted in the assay, the time interval of 20 min was found to be quite satisfactory for accurate determinations. Several hours would be required for the reaction to go to absolute completion. This is obviously undesirable for a rapid assay.

The reaction time of 20 min with ethanol is critical. If this time varies at all from 20 min, the assay no longer follows Beer's law. It is not known whether the effect of ethanol is caused by a chemical reaction with the 2,4-dinitrophenylhydrazone or is merely the result of the increased solubility of the derivative. Fig. 2 shows the influence of the time of reaction with ethanol.

The range of the method as presented here is between 0.25 and 3.0 μmoles 2-heptanone with an accuracy of ± 0.03 μmole. The assay follows Beer's law within the specified range. The extinction coefficient E or absorbancy index a_s for the assay for 2-heptanone is $1.375 \cdot 10^4$ ml/g·cm at 540 mμ.

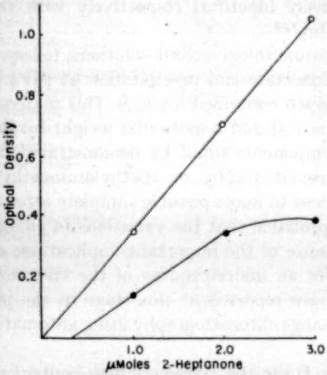

Fig. 1. The effect of ethyl alcohol on the assay for 2-heptanone. ○—○ 95% ethanol added; ●—● no ethanol added.

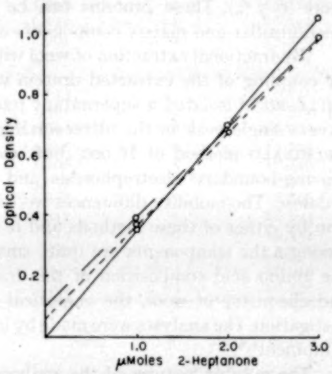

Fig. 2. The effect of time of reaction with ethanol on the assay for 2-heptanone. ○—○ reacted 20 min with ethanol; ○—○ reacted 15 min with ethanol; ○- - -○ reacted 25 min with ethanol.

For our purposes, all samples are steam distilled prior to assaying. It was found that this is the most satisfactory method for removing the ketone from the biological sytem. Filtration or centrifugation procedures were found to be unsatisfactory because of the extreme volatility of 2-heptanone. Furthermore, maximum production of methyl ketones by *Penicillium roqueforti* requires phosphate buffer and the steam distillation separates the ketone from the phosphate which is insoluble in ethanol and would thus interfere with the assay.

There is a loss of from 5–10% of this ketone during the distillation process; however, this loss is counteracted if the standard is also distilled. The rate of distillation must be controlled in order to obtain consistant results between samples. Exactly 4.0 ml of the distillate is collected at a rate of 30 drops/min.

Acetone may be assayed by this same procedure with no modification; the range of the method for acetone is the same as for 2-heptanone. The method also can be used for other ketones as well as keto acids and aldehydes. Slight modifications of the procedure may be required for some of these depending upon the biological system.

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Department of Bacteriology, University of Wisconsin, CHARLES W. HAIDLE
Madison, Wisc. (U.S.A.) S. G. KNIGHT

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Amino acid composition of a sulphur-rich protein from wool

Merino wool which contains about 3.5 % S appears to embody two main kinds of protein, one type having less sulphur than wool itself (1.5–2 %) and the other much more (6–7 %). These proteins can be tentatively identified respectively with the microfibrillar and matrix components of the fibre[1–5].

By fractional extraction of wool with potassium thioglycollate solutions, followed by coupling of the extracted protein with iodoacetate and precipitation at pH 4.1, GILLESPIE[6,7] isolated a supernatant fraction which contained 6.5 % S. This material gave a single peak in the ultracentrifuge (S_{20},w 1.5) and a molecular weight by the ARCHIBALD method of 16,000, but several components could be demonstrated by moving-boundary electrophoresis and by chromatography on diethylaminoethylcellulose. The mobility differences were insufficient to make possible complete separation by either of these methods and it seems probable that the variations in charge amongst the components are quite small. Because of the important implications of the amino acid composition of this fraction for an understanding of the structure and chemistry of wool, the analytical results are reported at this stage in the investigation. The analyses were made by ion-exchange chromatography using automatic equipment[8,9].

The notable features of the analysis (Table I) are the extremely high content of S-carboxymethyl cysteine, accounting for 29.4 % of the weight of the protein, and of serine, threonine and proline. The value for amide nitrogen (11 moles) suggests that aspartic (4 residues) and glutamic acids (12 residues) are largely present as their amides. Lysine is present only in small amount, about 1 residue/mole, whereas arginine is present to the extent of 9 residues/mole. These data show an excess of basic amino acid residues plus amide over aspartic plus glutamic acid residues of 4 residues/mole.

This calculation reveals the interesting fact that, in the native state in the wool fibre at pH 7, where the sulphur is in the form of –S–S–, these matrix proteins have a strong positive charge, and as the basic groups are almost entirely in arginine side chains the isoelectric point would be high, near the pK for the ionisation of tyrosine side-chain groups.

The low-sulphur[10] microfibrillar proteins with a negative charge at pH 7 are thought to be synthesised in the follicle whereas the matrix proteins are introduced into the fibre just below the zone of keratinization[3,4]. This conjunction of proteins of opposite charge may be very relevant to the early stages in fibre formation, keratinization providing additional cross-links at a later stage. The data may also throw light on the difference between the ortho and para segments of the Merino wool fibre, for if ROGERS'[2] view is correct, namely that these regions differ chiefly in containing different proportions of matrix and microfibrillar proteins, the *para* having more of the matrix than the *ortho*, then the microfibril-matrix complex of the *para* cortex will be less acidic than the corresponding complex in the *ortho* cortex. Certain aspects of the differing reactivity of side-chain groupings in wool may also be due to variations in the acidity of the proteins in their immediate environment.

If as seems likely, this high-sulphur fraction contains closely related molecular species, then the amino acid analysis indicates a molecular weight in the neighbourhood of 16,000 in agreement with the physico-chemical data.

TABLE I

	*µmoles/g***	*S.E.**	*Weight to contain 1 g equiv.*	*Estimated no. residues*	*Estimated mol. wt.*
Alanine	252	23	3,968	4	15,872
Amide	657	59	1,522	11	16,742
Arginine	509	22	1,965	8	15,720
Aspartic acid	255	6	3,921	4	15,684
S-carboxymethyl cysteine	1,604	36	623	27	16,831
Glutamic acid	724	18	1,381	12	16,572
Glycine	590	12	1,695	10	16,950
Histidine	68	8	14,705	1	14,705
Isoleucine	306	10	3,268	5	16,340
Leucine	332	12	3,012	6	18,072
Lysine	53	3	18,868	1	18,868
Phenylalanine	161	16	6,211	3	18,633
Proline	1,223	—	817	20	16,352
Serine***	1,281	28	780	21	16,392
Threonine***	1,003	16	997	17	16,949
Tyrosine	183	9	5,464	3	16,392
Valine	484	17	2,066	8	16,528
			Total	150	Mean 16,682

* S.E. = standard error of mean (3 observations).

** Mean of two 20-h and one 70-h hydrolyses in 6 *N* HCl at 110°.

*** Extrapolated values.

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Division of Protein Chemistry, J. M. GILLESPIE
C.S.I.R.O. Wool Research Laboratories,
Parkville N.2, Melbourne, Victoria (Australia)
Waite Agricultural Research Institute, University of Adelaide, D. H. SIMMONDS
Adelaide, South Australia (Australia)

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Effets des rayons u.v. sur la respiration des fragments d'amibes

Plusieurs auteurs ont essayé de mettre en évidence l'effet des radiations ionisantes[1–4] ou ultraviolettes[5,6] sur des fragments nucléés et anucléés de l'algue unicellulaire *Acetabularia mediterranea*. Leurs résultats sont concordants en ce qui concerne la radiosensibilité qui est plus grande pour les fragments sans noyau que ceux avec noyau.

L'un de nous (*Y.S.*)[7] a pu constater la même chose chez les fragments d'amibes en précisant cet effet général avec des tests cytochimiques et par autoradiographie et en concluant que les effets de l'irradiation se superposent à ceux de l'énucléation en ce qui concerne l'ARN, le glycogène et la synthèse de protéines.

La présente note est la suite de l'analyse des effets des rayons u.v. sur les fragments d'amibes, en choisissant cette fois la consommation d'O_2 comme point de comparaison.

Rappelons, d'une part, que les résultats des irradiations sur la respiration cellulaire de toute une série d'organismes sont discordants[8] et, d'autre part, que la respiration cellulaire est assez indépendante du noyau[9].

Il se pourrait, donc, que la consommation d'O_2 se comporte après l'irradiation différemment que l'ARN, le glycogène et la synthèse des protéines, sur lesquels le noyau a une influence importante.

La technique de culture, de section et d'irradiation des amibes est la même que pour le travail précédent[7]. La respiration a été mesurée grâce au microrespiromètre de LINDERSTRØM-LANG ET HOLTER. Le matin les amibes ont été coupées, irradiées et introduites dans les ludions. Entre l'irradiation et les premières mesures s'est écoulée environ 1 h. Il est important de souligner qu'une seule dose de 1200 ergs/mm^2 a été utilisée. En ce qui concerne les détails de la technique du ludion nous renvoyons au travail de LINDERSTRØM-LANG ET HOLTER[10]. Nos petits ludions à bouchon de verre sont une adaptation de ZEUTHEN[11] et GONSE[12]. A l'aide d'une pipette freinante calibrée on introduit sous la loupe dans l'ampoule, les amibes ou fragments (10 à 12 dans leur milieu de culture, liquide de Chalkey) et la bulle d'air. Cette dernière est spécifique pour chaque ludion; son volume (1 à 2 μl) a été mesuré lors de l'étude du "back ground" obligatoire pour chaque ludion neuf. Ensuite le reste de l'ampoule est rincé avec le milieu de flottaison de composition suivante: soude 0.05 N, détergent 0.5 %, solution saline isotonique à la soude.

Le travail a comporté 70 séries de mesures complètes, c. à d. faites au minimum pendant 6 h et d'heure en heure après l'introduction des ludions dans les ampoules de flottaison. Nous avons éliminé toutes les expériences qui semblaient défectueuses pour des raisons matérielles.

Le Tableau I indique les moyennes de mesures effectuées sur les fragments témoins et les fragments irradiés. En ce qui concerne les témoins on peut voir que les amibes entières consomment à peu près la même quantité d'O_2 que deux fragments nucléé et anucléé ensemble et que les anucléés consomment beaucoup moins que les nucléés, la différence étant significative ($P < 0.01$). Nos résultats diffèrent un peu de ceux de BRACHET. Chez lui les différences sont moins marquées, et les deux fragments consomment plus qu'une amibe entière. L'irradiation, tout de suite après la section, abaisse nettement la consommation d'O_2 chez les anucléées ($P < 0.01$), la chute étant de 47 %, mais n'a aucun effet sur les nucléés.

Abréviations: ARN, acide ribonucléique.

TABLEAU I

CONSOMMATION D'O_2 EN 10^{-3} μL/FRAGMENT/H

Entre parenthèses nombre d'expériences.

	Mesures faites tot de suite		*Mesures faites le lendemain*	
	Témoins	*Après irradiation*	*Témoins*	*Lendemain de l'irradiation*
Amibes entières	0.500 ± 0.010* (6)			
Fragments nucléés	0.310 ± 0.015 (8)	0.291 ± 0.016 (8)	0.280 ± 0.021 (5)	0.276 ± 0.019 (5)
Fragments anucléés	0.170 ± 0.008 (10)	0.099 ± 0.005 (10)	0.160 ± 0.013 (8)	0.123 ± 0.009 (10)

* Erreur type (S.E.).

Les mesures faites le lendemain de l'irradiation ont montré une situation identique: les anucléés irradiés consomment toujours moins que les témoins ($P < 0,05$), mais la perte n'est plus que de 23 %. Si dans ce cas on examine en détails les moyennes des 10 expériences, on constate que pour les 3 premières la consommation normale semble s'être rétablie, alors que pour les 7 suivantes elle reste diminuée de 37 %. Cette grosse différence est difficile à expliquer autrement que par une erreur technique des débuts.

Pour l'instant ces résultats cadrent parfaitement avec les autres résultats mentionnés au début du travail étant donné que les fragments nucléés continuent à respirer normalement après une irradiation de 1200 ergs/mm^2, et que les anucléés montrent une sensibilité très prononcée.

Institut de Biologie et Institut de Recherches Médicales,
Faculté de Médecine, Zagreb (Yougoslavie)

Y. ŠKREB
N. ŠKREB

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Activation of arylsulphatase B by chloride

The authors have reported the purification of an enzyme in ox liver hydrolysing *p*-nitrophenyl sulphate[1]. The action of this enzyme on this compound differed from arylsulphatase activities previously described in that it is dependent on the presence of chloride or certain other monovalent anions. However, the hydrolysis of nitrocatechol sulphate by the enzyme was not enhanced by chloride, but instead was partially inhibited by it. In the paper cited it was pointed out that the enzyme described had some similarities to the arylsulphatase B prepared from ox liver by Roy[2]. The latter enzyme has usually been studied with nitrocatechol sulphate as substrate, and it has been stated that it has a negligible activity towards *p*-nitrophenyl sulphate[3–6].

A direct comparison has now been made between the arylsulphatase of Webb and Morrow[1] and a specimen of arylsulphatase B made by the authors from the same batch of acetone-dried ox liver according to the directions of Roy[2]. The results of this comparison, which suggest that the enzymes are identical, are shown in Table I. The specific activities towards nitrocatechol and *p*-nitrophenyl sulphates were measured at pH 5.6 and 38° at arbitrary substrate concentrations of 8.3 mM and 2 mM respectively by the methods previously described, and are given relative to the absorbancy of the enzyme preparations at 280 mμ. The hydrolysis of *p*-nitrophenyl sulphate by arylsulphatase B also is strongly activated by chloride. Plots of rate of hydrolysis against chloride concentration give curves of similar shape for the two enzymes, and suggest chloride activation constants of the same order of magnitude, as shown in the table. The nitrophenylsulphatase activity of the two preparations shows similar sensitivity to phosphate and fluoride. The specific activity of the preparation of Webb and Morrow is more than double that prepared according to Roy.

TABLE I

A DIRECT COMPARISON OF TWO PREPARATIONS OF ARYLSULPHATASE

	Arylsulphatase B prepared according to Roy[2]	*Arylsulphatase prepared according to* Webb and Morrow[1]
Specific acitivity towards NCS at pH 5.6 in absence of chloride (μmoles/min/A_{280}).	0.035	0.0825
Specific acitivity towards NPS at pH 5.6 in absence of chloride (μmoles/min/A_{280})	0.0012	0.0024
Specific activity towards NPS at pH 5.6 in 0.17 M NaCl (μmoles/min/A_{280})	0.0046	0.013
Activity towards NPS at pH 5.6 in absence of chloride as a fraction of that in optimum chloride	0.26	0.185
Concentration of chloride giving half-maximal activity towards NPS (M)	0.015	0.021
Inhibition of NPS hydrolysis (in optimum chloride at pH 5.6) by $3.3 \cdot 10^{-4}$ M fluoride	60%	71%
Inhibition of NPS hydrolysis (in optimum chloride at pH 5.6) by $8.3 \cdot 10^{-3}$ M phosphate	66%	62%
Specific activity towards NPA at pH 5.6 in absence of chloride (μmoles/min/A_{280})	7.55	0.25
Ratio of activities: NPA:NCS:NPS (in chloride)	1640:7.6:1	19:6.4:1

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[*] Abbreviations, NCS, nitrocatechol sulphate; NPS, *p*-nitrophenyl sulphate; NPA, *p*-nitrophenyl acetate.

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The hydrolysis of 1.7 mM p-nitrophenyl acetate by the two enzymes has also been studied, in 0.167 M acetate buffer, pH 5.6, at 38°, by following the change of absorbancy at 525 mμ in the Beckman Ratio Recording Spectrophotometer DK-2. This hydrolysis is not affected by chloride, is not inhibited by phosphate at 10^{-3} M or less, and is 20 times more sensitive to fluoride than the hydrolysis of p-nitrophenyl sulphate by the enzymes ($pI_{50} = 4.7$). It is therefore probably due to a separate enzyme, present in considerable amounts in the preparation of arylsulphatase B, which is largely removed in the preparation of arylsulphatase by the method of WEBB AND MORROW. This arylesterase is at present being investigated.

Type I arylsulphatases (arylsulphatase C) and Type II (arylsulphatases A and B) have been distinguished partly by their relative activity towards p-nitrophenyl and nitrocatechol sulphates[5,6]. It can now be seen that the distinction is not as clearcut as the results previously obtained in absence of salt suggested, since in the presence of optimum chloride the activity of arylsulphatase B towards p-nitrophenyl sulphate is appreciable.

Department of Biochemistry, University of Cambridge
(Great Britain)
E. C. WEBB
PATRICIA F. W. MORROW

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Concerning "free" and "fixed" glycogen in rat liver

In recent years a good deal of discussion has developed on the nature and magnitude of the difference found when tissue glycogen is determined by the hot alkali (KOH) and by the cold trichloroacetic acid procedures. The significance of the problem arises from the likelihood that the glycogen left behind after extraction with trichloroacetic acid, the so-called "fixed" glycogen, is physiologically distinct from the "free" glycogen, that which can be extracted by trichloroacetic acid. For example, STETTEN, KATZEN AND STETTEN[1] have suggested that fixed glycogen may be glycogen which is bound to phosphorylase. Another possibility, in view of the recent work of LELOIR AND CARDINI[2], is that fixed glycogen might represent the "primer" required to initiate glycogen synthesis by the uridine diphosphate glucose pathway; this would imply that some fixed glycogen should remain intact even after total liver glycogen had been brought to very low levels by fasting.

Our concern with this question began when, in looking for an accurate method for determining liver glycogen in fasted rats, we found that there was no agreement in the literature on the magnitude of the fixed-glycogen fraction. In fact, there was serious question even as to the reality of fixed glycogen, since experiments of CARROLL, LONGLEY AND ROE[3] had shown that some of the material extracted by KOH and measured by anthrone was dialyzable, and could be decomposed on prolonged digestion in hot KOH. These workers also showed that repeated extraction with trichloroacetic acid led to a greater yield of glycogen than the single extraction generally used in this procedure[4], and concluded that there was really no difference between the amounts of glycogen obtained by KOH and by repeated extraction with trichloroacetic acid. However, that some glycogen remains even after exhaustive extraction with trichloroacetic acid has since been shown by STETTEN *et al.*[1] for livers with moderate to high glycogen content.

We began by repeating the experiments of CARROLL *et al.*[3] on the effect of prolonged digestion in hot KOH, but were unable to confirm their results, since neither with livers from fed nor from fasted rats did we find any signs of decomposition. Our subsequent experiments were then designed to test in as simple and direct a way as possible, whether or not there was a measurable difference in the amount of glycogen extracted by KOH and by trichloroacetic acid from livers with very low glycogen levels. At the same time, we wished to test the specificity of the KOH–anthrone method with low glycogen levels.

For the quantitative estimation of glycogen we relied on the criterion that glycogen should be that part of the alcohol-insoluble fraction of the extracts which on hydrolysis with acid yields glucose. To measure glucose we used the highly specific enzyme glucose oxidase*, essentially following the procedure of KESTON[5] and TELLER[6]. The reagent solution consisted of 12.5 mg glucose oxidase (Sigma "purified"), 5.0 mg horse-radish peroxidase (Worthington), and 1.0 ml 1 % *o*-dianisidine (Eastman) in methanol, dissolved in 100 ml water. Buffer was not used since the hydrolysates were brought to pH 7.0 with buffer before assaying. Equal volumes of reagent and glucose solution were mixed and left at room temperature for about 30 min. The reaction was stopped with a drop of conc. HCl. Readings were taken at 401 mμ on a Beckman Model DU Spectrophotometer. With each hydrolysate a control was run, using a reagent solution identical to that above except that glucose oxidase was left out; the amount of glucose present was calculated after subtracting this control value. Weight of glucose was converted to glycogen by multiplying by 0.9.

TABLE I

LIVER GLYCOGEN (mg/g WET WT.) OBTAINED BY KOH AND BY EXHAUSTIVE TRICHLOROACETIC ACID EXTRACTION, AS DETERMINED BY GLUCOSE OXIDASE AND ANTHRONE PROCEDURES

KOH method *Digestion time (h)*	*Glucose oxidase*		*Anthrone*	
0.25	0.39	0.34	0.59	0.55
1	0.34	0.32	0.47	0.49
4	0.31	0.35	0.50	0.53
8	0.34	0.31	0.60	0.59
24	0.34	0.32	0.56	0.57
Trichloroacetic acid method	0.16	0.19	0.21	0.20

The results of a typical experiment are given in Table I. For this experiment the livers of three fasted male rats were pooled, minced and mixed thoroughly. A part was taken for KOH and a part for trichloroacetic acid extraction. KOH extraction and alcohol precipitation was carried out by the method of GOOD, KRAMER AND SOMOGYI[7], except that the digestion times were those given in the first column. Trichloroacetic acid extraction was by the method of CARROLL *et al.*[3], with four extractions; all steps were carried out at less than 5°. Glycogen was precipitated by adding 1.4 vol. ethanol (rather than 5 vol.[3], since, if anything, better yields were obtained with 1.4 vol.) along with a pinch of LiBr. Duplicate samples were taken throughout. The method of SEIFTER *et al.*[8] was used for the anthrone determinations. The glucose oxidase determination was carried out as described above, after the glycogen had been hydrolysed at 100° in approx. 0.6 *N* HCl for 2.5 h, neutralized with NaOH and brought to pH 7.0 with phosphate buffer. Glucose standards were treated in the same way.

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* We are indebted to Dr. D. NORMAN for suggesting to us the use of this enzyme.

The results in Table I, typical of several such experiments, show that there is no significant decrease in the recovery of glycogen after prolonged digestion in KOH. The anthrone values decrease slightly and then increase; in some experiments they have increased and then remained constant. This erratic behavior is undoubtedly due to non-glycogen, anthrone-sensitive material[3], which is represented here by the difference between the anthrone and glucose oxidase measurements. From scattered observations it appears that this difference amounts to 0.1–0.2 mg glycogen/g tissue, regardless of the glycogen content.

The main point brought out in Table I is that, even when a highly specific method for glycogen is used, in livers with very low glycogen levels there is a clear difference between the amount of glycogen obtained by KOH extraction and by exhaustive extraction with trichloroacetic acid. This finding supports the validity of the distinction generally made in the literature between free and fixed glycogen, and is consistent with hypotheses on the nature of fixed glycogen that require this fraction to be present even in animals with very low liver glycogen levels.

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Worcester Foundation for Experimental Biology,
Shrewsbury, Mass. (U.S.A.)
S. B. KORITZ*
A. MUNCK**

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* Present address: Department of Biochemistry, School of Medicine, University of Pittsburgh, Pittsburgh, Penn. (U.S.A.).

** Present address: Department of Physiology, Dartmouth Medical School, Hanover, N.H. (U.S.A.).

Interaction of one mole of copper with the alpha amino group of bovine serum albumin

There have been repeated reports of a tight complexing of 1–1.5 moles of copper by bovine serum albumin, by a mechanism which apparently does not involve the albumin's single sulfhydryl group[1–5]. KOLTHOFF AND WILLEFORD in a short communication[6] suggested the possibility that the site of this interaction is the α-amino group of the single N-terminal aspartyl residue, but have not subsequently presented evidence in support of this concept[1].

In the course of studies involving the dialysis of bovine serum albumin against large volumes of water[7], it was noted that the copper content of the albumin increased while the yield of N-terminal DNP-aspartic acid decreased. The decrease in yield of DNP-aspartic acid was approximately equal to the molar amount of copper bound, as shown in Table I. The source of the copper was doubtless the tanks in which the freshly-distilled water was stored.

TABLE I

EFFECT OF DIALYSIS ON α-AMINO GROUP AND COPPER CONTENTS OF SERUM ALBUMIN

	Undialyzed albumin	*Dialyzed albumin*
DNP-aspartic acid[8] (mole/mole protein, corr.)	0.81	0.37
Copper[9] (mole/mole protein)	0.0	0.55

The stoichiometry of the copper interaction was investigated by addition of $CuSO_4$ to 8 % solutions of bovine serum albumin (Armour's crystalline Bovine Plasma Albumin, lot No. – T68412) at pH 6. After 1 h at 23°, solid $NaHCO_3$ was added and the yield of N-terminal aspartic acid determined.

Addition of increasing amounts of copper (Fig. 1) caused a linear and approximately equimolar drop in the free alpha amino group. The minimum value was reached at 0.9 mole copper/mole protein. However, addition of 25 moles ethylenediaminetetraacetic acid/mole protein instead of copper gave a slight increase (0.1 mole/mole) in the yield of DNP-aspartic acid, suggesting that about 10 % of the α-amino group of the untreated albumin may have been combined with a metal, perhaps nickel[1], since no copper was detected by analysis.

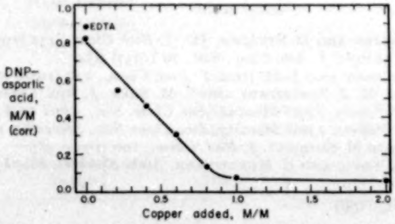

Fig. 1. Yield of DNP-aspartic acid from bovine serum albumin with addition of increasing amounts of $CuSO_4$. Each point is the average of 5 determinations. The point labeled "EDTA" represents the addition of 25 moles ethylenediaminetetraacetic acid/mole protein.

Abbreviation: DNP-, dinitrophenyl-.

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The bound copper was not removed upon lyophilization, heating, or treatment with 8 *M* urea. However, the copper could be removed, and the expected yield of DNP-aspartic acid obtained, by precipitation of the Cu-albumin with trichloroacetic acid.

On the basis of these results, it is suggested that the site of binding of the first mole of copper by bovine serum albumin is, at least in part, the free α-amino residue of the terminal aspartic acid. The binding is strong enough to resist attack by fluorodinitrobenzene at pH 9. Since free aspartic acid does not bind copper with the avidity shown by albumin[1], the copper probably interacts also with other groups which are present in the proper spatial relationship.

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The Mary Imogene Bassett Hospital (Affiliated with Columbia University) T. PETERS, Jr.
Cooperstown, N.Y. (U.S.A.),
Chemistry Department, Carlsberg Laboratory, Copenhagen (Denmark)

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Spectrophotometric studies on the binding of acridine orange to ribonucleic acid and deoxyribonucleic acid

BEERS, HENDLEY AND STEINER[1] have reported the formation of two types of complexes between acridine orange and polyadenylic acid in solution. The dye–acid complex I was formed when the relative concentration of the acid to the dye was low and it showed an absorption spectrum with a peak at 465 mμ. The complex II observed at relatively high concentration of the polymer acid (ratio of its bases to dye 100:1) was associated with a different absorption spectrum with a peak at 502 mμ and strong fluorescence in the green. Working with ribonucleic acid and deoxyribonucleic acid obtained from *Micrococcus lysodeikticus*, these authors observed that

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Abbreviations: AO, acridine orange; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

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acridine orange formed a mixture of complexes I and II with RNA and only complex II with DNA. On the basis of these observations they have suggested that the failure of DNA to form complex I would provide a highly specific method for determination of DNA/RNA ratio in the mixture of these two nucleic acids. We have been carrying out similar experiments on the binding of AO to RNA and DNA and some of our results are reported here.

The acridine orange used in these experiments was "Grubler" stain manufactured by Chroma-Gesellschaft Schmid and Co., Stuttgart. The RNA was supplied by Nutritional Biochemical Corporation, Cleveland, U.S.A. The DNA from calf thymus was obtained from Dr. J. A. V. BUTLER's Laboratory, Chester Beatty Research Institute, London. The spectral absorption results were measured on a Beckman DU Spectrophotometer at room temperature (22°–25°). The binding studies were conducted at two different concentrations of AO viz. 0.001 % and 0.005 % at pH 7.4 for DNA and at pHs 6.3, 7.4 and 10.3 for RNA.

The changes observed in the spectral absorption of the dye (0.001 %) with varying amounts of RNA and DNA in solution at pH 7.4 are shown in Fig. 1. No qualitative differences in the formation of complexes I and II are seen between RNA and DNA. At relatively low concentrations of nucleic acid to the dye, the effect in both cases is to suppress the intensity of the AO band at 490 mμ. When the relative concentration of the acid goes above a certain critical range, the formation of complex II shown by a strong band at 502 mμ is evident in the mixture of AO and RNA as well as in that of AO and DNA. Both the solutions exhibited strong green fluorescence, whenever the formation of complex II was indicated from the spectral absorption data. The observations at higher concentration of the dye (0.005 %) are shown in Fig. 2. Allowing for some quantitative variation the results are essentially of the same nature.

PEACOCKE AND SKERRETT[2] found two types of effects in their spectrophotometric study of the interaction of acridine derivatives with nucleic acids. They observed that the peak of the characteristic band of acridine derivatives was displaced to a higher wavelength in the mixtures of proflavine with DNA, acid-treated DNA, RNA and purine deoxyribonucleotides and in mixtures of 5-amino-acridine with DNA. On the other hand, only lowering of over-all intensity of the same band, with no shift in the peak wavelength was noted by them for the mixtures of proflavine with apurinic acid and with 5-amino-1,2,3,4-tetrahydroacridine with DNA. In the present work with AO, it appears that while the principal effect of the formation of complex I is to suppress the AO band at 490 mμ without much affecting its neighbouring band at 460 mμ, the effect of formation of complex II is to shift the 490 mμ band to 502 mμ. In such a case it would not be necessary to expect a clear peak at 460 mμ in order to indicate the presence of complex I. The appearance of a distinct peak at 460 mμ would depend upon (a) the relative intensities of the 460 mμ and 490 mμ bands of AO, a factor which is sensitive to pH and concentrations of AO as shown by ZANKER[3], and (b) the relative rates of formation of complexes I and II which determine the maximum depression at 490 mμ through formation of complex I, before the formation of complex II increases the spectral absorption in the same region. In our results there is a clear indication of suppression of the 490 mμ peak of AO at relatively low concentrations of DNA and of a displacement of wavelength when the critical range of the relative concentration of the acid has been exceeded. The same is also true for RNA. These observations are in conformity with those of

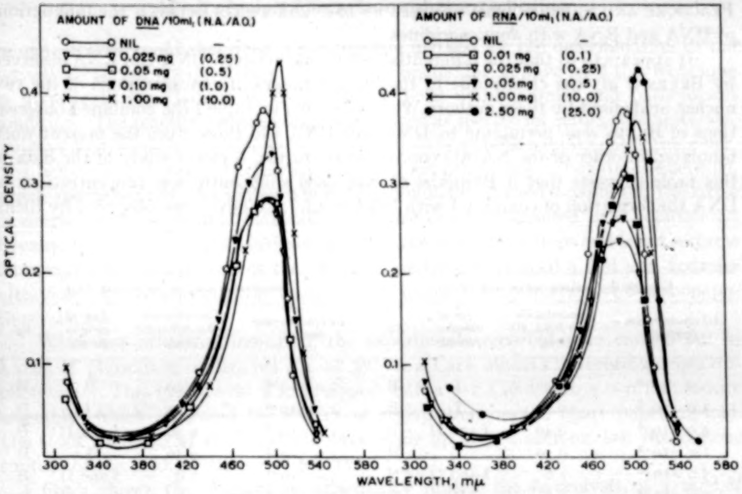

Fig. 1. Absorption spectra of acridine orange (concentration 0.001 %) in veronal buffer, pH 7.4, with the admixture of nucleic acids.

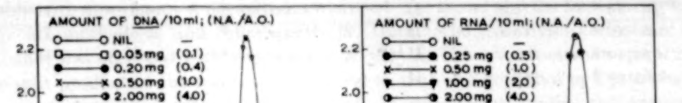

Fig. 2. Absorption spectra of acridine orange (concentration 0.005 %) in veronal buffer, pH 7.4, with the admixture of nucleic acids.

Peacocke and Skerret which showed no basic differences between the interactions of DNA and RNA with aminoacridines.

It appears to us that the differential behaviour between DNA and RNA observed by Beers *et al.* was chiefly due to the limited ranges of concentrations of the two nucleic acids used by these authors. We have shown in Table I the combined observations of Beers *et al.* pertaining to DNA and RNA and those from the present work tabulated in order of the NA/AO concentration ratios. A closer study of the data in this table suggests that if Beers *et al.* had used sufficiently low concentrations of DNA the formation of complex I with DNA would also have been observed by them.

TABLE I

Complexes of acridine orange with DNA				*Complexes of acridine orange with RNA*			
AO concentration (mg/ml)	*Concentration ratio DNA/AO (w/w)*	*Type of complex formed*	*Ref.*	*AO concentration (mg/ml)*	*Concentration ratio RNA/AO (w/w)*	*Type of complex formed*	*Ref.*
$5.0 \cdot 10^{-2}$	0.10	I	B	$1.0 \cdot 10^{-2}$	0.10	I	B
$1.0 \cdot 10^{-2}$	0.25	I	B	$1.0 \cdot 10^{-2}$	0.25	I	B
$5.0 \cdot 10^{-2}$	0.40	I and II	B	$1.0 \cdot 10^{-2}$	0.50	I and II	B
$1.0 \cdot 10^{-2}$	0.50	I	B	$5.0 \cdot 10^{-2}$	0.50	I	B
$5.0 \cdot 10^{-2}$	1.0	I and II	B	$5.0 \cdot 10^{-2}$	1.0	I and II	B
$1.0 \cdot 10^{-2}$	1.0	II	B	$5.0 \cdot 10^{-2}$	2.0	I and II	B
$1.35 \cdot 10^{-3}$	2.26	II	A	$5.0 \cdot 10^{-2}$	4.0	I and II	B
$1.35 \cdot 10^{-3}$	4.53	II	A	$1.35 \cdot 10^{-3}$	8.15	I and II	A
$5.0 \cdot 10^{-2}$	8.0	II	B	$1.0 \cdot 10^{-2}$	10.0	II	B
$1.0 \cdot 10^{-2}$	10.0	II	B	$5.0 \cdot 10^{-2}$	20.0	II	B
$1.35 \cdot 10^{-3}$	340	II	A	$1.0 \cdot 10^{-2}$	25.0	II	B
$2.65 \cdot 10^{-3}$	517	II	A				

A, Beers, Hendley and Steiner[1]; B, this work.

The fluorescence shift from green towards red[4] shown by AO-stained chromatin matter of the cells exposed to an X-ray dose above about 2000–3000 R suggests that in the chromatin there exists some weakly bound structural lattice which keeps the local DNA concentrations so high that acridine orange can form complex II with it and at the critical dose of X-radiation this lattice is dissolved or so damaged that the local DNA concentrations are reduced and in consequence the equilibrium for AO–DNA binding is shifted from complex II to complex I.

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Biophysics Department, Indian Cancer Research Centre,
Bombay (India)
N. S. Ranadive
K. S. Korgaonkar

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Hydrolysis of β-carbonaphthoxycholine

In 1951 RAVIN *et al.*[1] described a colorimetric method for the determination of the activity of serum cholinesterase. Several authors (*cf.* ref. 2) have used this technique to investigate the changes in cholinesterase level under normal and pathological conditions. The reaction, which was characterized as specific for serum cholinesterase, employs β-carbonaphthoxycholine as substrate. When cholinesterase splits this ester, it is believed that β-naphthylcarbonic acid (β-naphthylmonocarbonate) is liberated, which spontaneously and immediately decarboxylates to yield β-naphthol. The latter is coupled with a suitable diazonium salt and can be measured in a colorimeter. Because the original method includes several time-consuming steps and is not suitable for assaying the initial rates of the reaction, we have developed a fast and accurate ultraviolet spectrophotometric technique to follow the hydrolysis of β-carbonaphthoxycholine.

When not otherwise indicated, the experiments reported were carried out in a 0.05 *M* phosphate buffer, pH 7.3, at 37° in a Cary Model 11 Recording Spectrophotometer. This instrument was equipped with a 0–2.4 and with a 0–0.1 slide-wire assembly, which increased the extent of registration on the chart paper 24-fold. The stock solutions of the substrate were made up in 5 % acetone, but the acetone content of the final dilutions was less than 1 %.

Fig. 1 shows the changes in absorbancy during the hydrolysis of $1 \cdot 10^{-4}$ *M* β-carbonaphthoxycholine by a Cholase solution diluted 1:4000 (v/v). The ultraviolet range of the Cary spectrophotometer was scanned at a rate of 10 Å/sec. A slide-wire assembly with absorbancy range of 0–2.4 was used. The initial and the final absorption spectra are marked (a) and (b) respectively. During the hydrolysis an increase in absorbancy was observed with a maximum at 3275 Å. The u.v.-absorption spectrum of the split product of the reaction is identical to that of β-naphthol in a phosphate buffer containing Cholase and $1 \cdot 10^{-4}$ *M* KI. In subsequent studies the enzymic hydrolysis of β-carbonaphthoxycholine was measured at 3250 Å. At the range of concentrations employed, β-naphthol obeyed Beer's law. With low concentrations of the substrate, the more sensitive 0–0.1 slide-wire assembly was used.

The MICHAELIS constant of the hydrolysis of β-carbonaphthoxycholine by Cholase was found to be $1 \cdot 10^{-4}$ *M*.

Table I summarizes the effect of various inhibitors on the enzymic hydrolysis of β-carbonaphthoxycholine. Human serum and Cholase both hydrolyze this substrate. Eserine in 10^{-5} *M* concentrations did not inhibit more than 89 % of the activity of human serum cholinesterase. That the activity remaining after the incubation with eserine was not due to serum aryl (aromatic) esterase was shown by the fact that EDTA did not influence the hydrolysis of β-carbonaphthoxycholine. It was demonstrated by ERDÖS *et al.*[3] that EDTA inhibits this esterase. Furthermore, Fraction IV-1 of human plasma, which contains an aryl esterase[4], did not hydrolyze β-carbonaphthoxycholine to any significant extent. TPA, which accelerates plasma cholinesterase

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Abbreviations: Cholase, a preparation of concentrated, purified, human plasma cholinesterase made by Cutter Laboratories. The stock solution used contained 35 mg/ml; R.B.C. cholinesterase, purified bovine red-blood-cell "true" cholinesterase made by Winthrop Labs.; EDTA, ethylenediaminetetraacetic acid, disodium salt; Mytelase, N,N′-bis(2-diethylaminoethyl)-oxamine bis-2-chlorobenzylchloride; TPA, Tetra-*n*-propylammonium iodide; snake venom, dried venom of *Najah hannah*.

with aromatic substrates[5], also enhanced the hydrolysis of β-carbonaphthoxycholine. Certain features of the mechanism of this acceleration[6] have been discussed elsewhere.

Several publications[1,2] indicated that β-carbonaphthoxycholine is a specific substrate of serum "pseudo" cholinesterase. In the present studies it was found that R.B.C. cholinesterase hydrolyzes this substrate also but at a slower rate. Snake venom, which contains the same enzyme, was similarly active. The enzymes in these two preparations were inhibited by a specific inhibitor of "true" cholinesterase, Mytelase

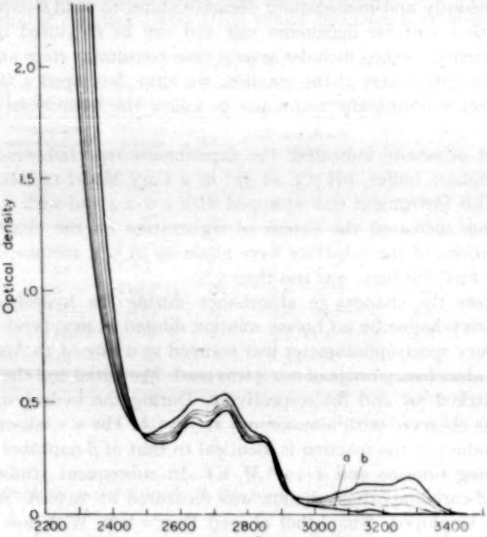

Fig. 1. Continuous scanning of the hydrolysis of β-carbonaphthoxycholine by Cholase.

TABLE I

HYDROLYSIS OF β-CARBONAPHTHOXYCHOLINE IODIDE BY CHOLINESTERASES

Pooled, normal human serum was used in a final dilution of 1:50 (v/v). Eserine and Mytelase were preincubated with the enzymes for 20 min. The concentration of the substrate was $5 \cdot 10^{-4}$ *M*. The rates were corrected for non-enzymic hydrolysis.

Source of enzyme	*Hydrolysis rate*	*Change in activity (%) in the presence of:*			
		EDTA ($1 \cdot 10^{-3}$ *M*)	*Eserine* ($1 \cdot 10^{-5}$ *M*)	*TPA* ($1 \cdot 10^{-3}$ *M*)	*Mytelase* ($2.5 \cdot 10^{-7}$ *M*)
Human serum	14.3*	0	— 89	+ 62	0
Cholase	42.8**	—	— 96	+ 86	0
Snake venom	0.8**	—	—100	—	— 91
R.B.C. cholinesterase	1.1**	—	— 94	— 56	— 94

* μmoles/ml/30 min.
** μmoles/mg/30 min.

(see Table I). Mytelase in the same concentration did not influence the activity of the "pseudo" cholinesterase. In contrast to its behavior with plasma cholinesterase, TPA inhibited the R.B.C. cholinesterase.

Like these enzymes, imidazole and histidylhistidine liberate β-naphthol from β-carbonaphthoxycholine. Kinetic experiments were carried out with the catalyst in excess and pseudo-first-order kinetics were observed. Plots of logarithm of the per cent of remaining unhydrolyzed ester against time gave straight lines up to about 95 % of reaction (see for example Fig. 2). Mean values of the first-order constants for the two catalysts employed were 0.034 min^{-1} for histidylhistidine, and 0.028 min^{-1} for imidazole. Imidazole and histidylhistidine catalyze the hydrolysis of β-naphthylacetate also. On the other hand, imidazole does not hydrolyze acetylcholine[7]. These findings taken with the other results would suggest that these catalysts attack the naphthoxy–carbonyl bond of β-carbonaphthoxycholine rather than the choline–carbonyl bond. In contrast, the enzymic attack on the same substrate is at the choline–carbonyl bond.

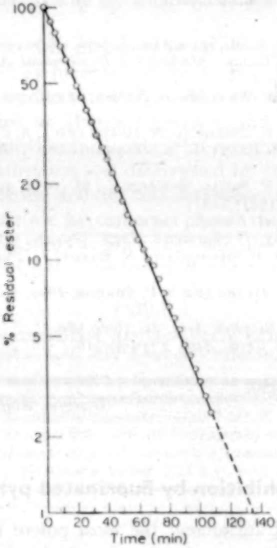

Fig. 2. Plot showing the hydrolysis of β-carbonaphthoxycholine ($1 \cdot 10^{-3}$ *M*) catalyzed by histydylhistidine ($1 \cdot 10^{-2}$ *M*) in a $7.5 \cdot 10^{-2}$ *M* phosphate buffer.

HOFSTEE[8] was the first to use the u.v. spectrophotometric technique in conjunction with studies of esterases. KALOW[9] described a u.v. method for serum cholinesterase which was later modified[10] for a recording spectrophotometer. It seems to us that the advantage of the present method over the others[11–14] is that a commercially readily available substrate is used with relatively high dilution of human serum at a wavelength where little or no optical interference is expected from diluted serum proteins. Furthermore, the mechanism of hydrolysis of esters of β-naphthol by imidazole and other compounds could be studied with the same technique.

EDTA was kindly supplied by Geigy Chemicals, Mytelase by Winthrop Laboratories, and human serum by Dr. M. P. WESTERMAN of the University of Pittsburgh Medical School. The authors gratefully acknowledge the help of Drs. S. JOHNSON and J. G. PRITCHARD.

E. G. ERDÖS
Mellon Institute, Pittsburgh, Pa. (U.S.A.) C. R. DEBAY

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Feedback inhibition by fluorinated pyrimidines

The fluorinated pyrimidines are among the most potent inhibitors of nucleic acid biosynthesis yet developed[1]. Three mechanisms by which 5-fluoropyrimidine analogues may interfere with pyrimidine metabolism have been extensively studied by HEIDELBERGER *et al.*[2,3]. (a) Interference with the methylation of deoxyuridylic acid to form thymidylic acid, presumably by 5-fluoro-2-deoxyuridine monophosphate. (b) Inhibition of the conversion of free pyrimidine bases (uracil and orotic acid) to RNA ribonucleotides. For 5-fluoroorotic acid this may include inhibition of orotidylic acid formation[4]. (c) Production of an abnormal RNA containing fluorinated pyrimidine bases. Indirect evidence from studies with Ehrlich ascites-carcinoma cells suggests that the first of these mechanisms is of the greatest quantitative importance in inhibition of tumor growth. In summary this represents inhibition by the abnormal pyrimidine base *per se*, inhibition by abnormal nucleotides formed biosynthetically from the base, and possibly more subtle inhibition exerted by the resulting abnormal nucleic acids. Recently there has been considerable interest in yet another mechanism by which structural analogues may serve as antimetabolites—that of inhibiting the synthesis of the corresponding natural product[5,6]. Here the structural analogue seems to function actively in a normal negative-feedback control mechanism in the cell,

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Abbreviation: RNA, ribonucleic acid.

which ordinarily serves as a "chemostat" to regulate the rate of synthesis of the natural product[7]. In this way the analogue not only influences the fate of a substance but also its rate of formation. YATES AND PARDEE have previously reported that orotic acid is an inhibitor of the cyclization of carbamylaspartic acid to dihydroorotic acid by dihydroorotase and that cytidine 5′-phosphate and cytidine competitively inhibit aspartate carbamyltransferase[8]. It seemed of interest to determine if 5-fluoroorotic acid and 5-fluorocytidine were also active in these systems. Similar studies were also carried out on 6-uracil methyl sulfone, another competitive inhibitor of orotic acid metabolism[9].

Dihydroorotase activity was measured in sucrose extracts of *E. coli* B sonicates by the conversion of [^{14}C]carbamylaspartate to dihydroorotate during a 30-min incubation at 30° at pH 5.5 (acetate buffer). The product was isolated with carrier dihydroorotic acid, purified on Dowex 1-formate, and measured for dihydroorotic acid and radioactivity as previously described[10]. From the specific activity the amount of dihydroorotic acid synthesized was determined by the standard carrier formula. Aspartate carbamyltransferase activity was determined in *E. coli* B extracts by the carbamylation of [^{14}C]aspartate by carbamyl phosphate to form carbamylaspartate, which was isolated and measured as previously described[10].

Table I illustrates that 5-fluoroorotic acid was found to be considerably more

TABLE I

INHIBITION OF DIHYDROOROTASE AND ASPARTATE CARBAMYLTRANSFERASE BY 5-FLUOROOROTIC ACID AND 5-FLUOROCYTIDINE, RESPECTIVELY

The assay of dihydroorotase was carried by incubation at 30° for 30 min in a final volume of 1 ml: [^{14}C]carbamylaspartate ($3.3 \cdot 10^5$ counts/min/μmole), $2.5 \cdot 10^{-4}$ *M*; sodium acetate buffer (pH 5.5), 0.125 *M*; ethylenediaminetetraacetate, $5 \cdot 10^{-3}$ *M*; *E. coli* B extract, 0.17 mg *N*; inhibitors as noted. The assay of aspartate carbamyltransferase was carried out at 37° for 30 min in a volume of 1 ml: [^{14}C]-L-aspartate ($9.3 \cdot 10^4$ counts/min/μmole), $5 \cdot 10^{-3}$ *M*; carbamyl phosphate, $5 \cdot 10^{-3}$ *M*; $MgCl_2$, $5 \cdot 10^{-3}$ *M*; phosphate buffer (pH 6.5), 0.25 *M*; *E. coli* B extract, 0.3 mg N; inhibitors as noted.

A. Dihydroorotase	*Concn.* *M*	*Dihydroorotic acid formed (mμmoles)*	*Inhibition %*
Control		100	0
Orotic acid	$0.5 \cdot 10^{-3}$	98	2
	$2 \cdot 10^{-3}$	95	5
	$4 \cdot 10^{-3}$	84	16
5-fluoroorotic acid	$0.5 \cdot 10^{-3}$	83	17
	$2 \cdot 10^{-3}$	42	58
	$4 \cdot 10^{-3}$	30	70
6-uracil methyl sulfone	$0.5 \cdot 10^{-3}$	96	4
	$2 \cdot 10^{-3}$	97	3
	$4 \cdot 10^{-3}$	96	4

B. Aspartate carbamyltransferase	*Concn.* *M*	*Carbamylaspartic acid formed (mμmoles)*	*Inhibition %*
Control		302	0
Cytidine	$1 \cdot 10^{-2}$	247	18
	$2 \cdot 10^{-2}$	163	46
Cytidine 5′-phosphate	$2 \cdot 10^{-2}$	130	57
5-fluorocytidine	$0.5 \cdot 10^{-2}$	249	18
	$1 \cdot 10^{-2}$	134	56
5-fluoroorotic acid	$0.5 \cdot 10^{-2}$	272	10

active than orotic acid as an inhibitor of the cyclization of carbamylaspartate to dihydroorotate by dihydroorotase. The inhibition was competitive with a calculated K_i of $1.5 \cdot 10^{-3}$ M (Fig. 1). In this system 6-uracil methyl sulfone was inactive as a feedback inhibitor despite its previously demonstrated effectiveness as a competitive inhibitor of orotic acid in the orotidine 5′-phosphate pyrophosphorylase reaction[9]. Feedback inhibition of aspartate carbamyltransferase, the first enzyme unique to pyrimidine biosynthesis, was shown with cytidine and cytidine 5′-phosphate (Table I) in confirmation of previous studies[8]. 5-Fluorocytidine was found to be approximately twice as active as cytidine or cytidine 5′-phosphate as an inhibitor in this system. The limited amount of 5-fluorocytidine available precluded kinetic studies. By analogy with cytidine the fluoro analogue probably served as a competitive inhibitor primarily of carbamyl phosphate[8]. 5-Fluoroorotic acid was not an effective inhibitor of aspartate carbamyltransferase.

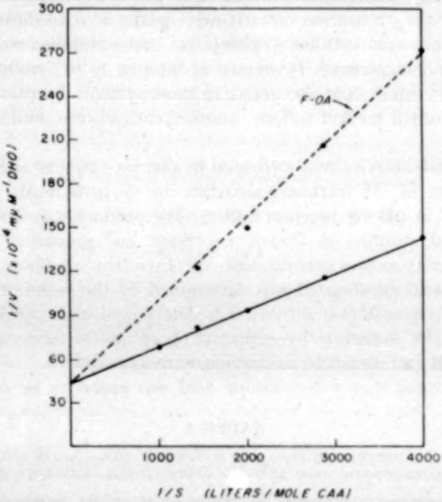

Fig. 1. LINEWEAVER-BURKE plot of the inhibition of dihydroorotase by 5-fluoroorotic acid ($2 \cdot 10^{-3}$ M). DHO, dihydroorotic acid; CAA, carbamylaspartic acid; F-OA, 5-fluoroorotic acid.

The studies described here demonstrate two additional ways in which the 5-fluorinated pyrimidines may inhibit nucleic acid synthesis. The quantitative importance of these reactions *in vivo* cannot be evaluated from the data available. Relatively high concentrations of the analogues were used in these *in vitro* studies. The inhibition is competitive, however, so that the steady-state concentrations *in vivo* of the normal metabolites (carbamylaspartate and carbamyl phosphate) control the inhibitory activities of the analogues. Information concerning the relative intracellular concentrations of the fluoro analogues and their normal competitors is not yet available during periods of carcinostatic use. With this reservation in mind the diagram of BOSCH *et al.*[3] suggesting the probable sites of action of the fluorinated pyrimidines can be extended as shown in Fig. 2. The results reported here document further examples of the action of antimetabolites as feedback inhibitors of biosynthesis.

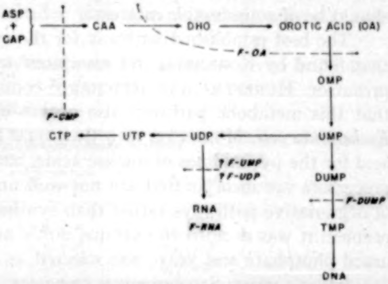

Fig. 2. Diagram showing sites of the inhibitory action of the fluorinated pyrimidines. ASP, L-aspartate; CAP, carbamyl phosphate; CAA, carbamylaspartate; DHO, dihydroorotate; OMP, orotidine 5′-phosphate; UMP, UDP, UTP, uridine mono-, di-, and triphosphate; CTP, cytidine triphosphate; DUMP, deoxyuridine 5′-phosphate; TMP, thymidine 5′-phosphate; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

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Department of Medicine, Mass. General Hospital and Harvard Medical School, Boston, Mass. (U.S.A.)

L. H. SMITH, JR.
M. SULLIVAN

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Studies on the incorporation of orotic acid into the 5-ribosyluracil phosphate of the ribonucleic acids of yeast

5-Ribosyluracil phosphate was first isolated from the soluble ribonucleic acids of yeast by DAVIS AND ALLEN[1]. This nucleotide is readily distinguished from the other nucleotides of ribonucleic acids by a shift from 263 mμ to 286 mμ in the maximum of its u.v.-absorption spectrum when an acidic or neutral solution which contains it is made alkaline. Since the first reported existence of this nucleotide, it has been found in a variety of tissues from plants and animals, and in microorganisms[2,3]. YU AND ALLEN[2], SCANNELL, CRESTFIELD AND ALLEN[4], and COHN[5] succeeded in establishing

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the structure of the nucleoside as that of an isomer of uridine in which the ribose is attached to position 5 of uracil. It is noteworthy that this is a carbon linkage rather than the common glycosidic linkage to the nitrogen at position 3, hence its biosynthesis should be of considerable interest.

The best established pathway for the synthesis of uridylic and cytidylic acids is that found by KORNBERG and associates[6] in yeast in which orotic acid is used as a precursor. HURLBERT AND REICHARD[7] confirmed KORNBERG's discovery and found that this metabolic pathway also occurs in tissues from birds, mammals, and in *Escherichia coli*. MOKRASCH AND GRISOLIA[8] have found precursors other than orotic acid for the pyrimidines of nucleic acids, but the metabolic pathways by which these precursors are incorporated are not well understood and may involve the reversal of degradative pathways rather than synthesis *de novo*. As the first approach to the problem it was decided to examine orotic acid as a possible precursor for 5-ribosyluracil phosphate and yeast was selected as a convenient organism for this purpose.

Baker's yeast, *Saccharomyces cerevisiae*, was grown in 4 l of Williams solution[9,10] to which had been added 23 mg of [6-^{14}C]orotic acid containing 0.2 mC of ^{14}C. 40 g yeast were used as an inoculum. The culture was aerated vigorously at room temperatures for 10 h and the yeast was harvested by centrifugation. 189 g of wet yeast were obtained.

The ribonucleic acids were isolated and purified by the use of the method of CRESTFIELD, SMITH AND ALLEN[11]. In this procedure the ribonucleic acids are fractionated by making the solution 1 *M* with respect to NaCl. Most of the ribonucleic acids precipitate but about 15 % remain in solution. Since the fraction which is soluble in 1 *M* NaCl has been shown to contain a larger quantity of 5-ribosyluracil than does the insoluble fraction, the investigation was conducted using the soluble fraction. In order to obtain the nucleotide components of the soluble ribonucleic acids, 200 mg of the soluble ribonucleic acids were dissolved in 50 ml 1 *M* piperidine and hydrolyzed at 55° for 48 h[12]. After the first 24 h an additional 4.5 ml of redistilled piperidine was added to the mixture to insure complete hydrolysis. This hydrolytic procedure was tested against a sample of commercial cytidylic acid and found not to produce any significant quantity of deamination to uridylic acid. Upon completion of hydrolysis, the mixture was evaporated to dryness in a rotary evaporator and redissolved in 10 ml water. A portion of this solution which contained 20 mg of nucleotides was adsorbed on a Dowex-1-formate column (0.88 cm^2 × 16 cm) and eluted following the procedure of COHN AND VOLKIN[13] for the separation of nucleotides. Ammonium formate was substituted for sodium formate in the eluting system so that the salt could be sublimed by lyophilization. The elution of the nucleotides from the column was followed by their absorption at 260 mμ as determined in the Beckman DU spectrophotometer. 5-Ribosyluracil phosphate emerged as a peak immediately preceding that of uridylic acid. The pyrimidine nucleotides were freed from salt and water by lyophilization and submitted to paper chromatography in an isopropanol–acetic acid–water mixture (60 : 30 : 10) for further purification[1]. After paper chromatography the u.v.-absorbing bands were cut from the paper, eluted to tips, and dissolved. Absorption at 250, 260, 280, and 290 mμ was determined. Spectral ratios were calculated to insure the purity of the solutions, and concentrations were calculaed from the absorbancy at 260 mμ.

Aliquots of each nucleotide were plated on three planchets and dried under vacuum. Each planchet was counted three times to 1280 counts in an automatic gas-flow counter. The values were averaged and converted to counts/min and specific activities.

In one experiment the purine nucleotides were also collected, purified, and counted. They had no measurable activity.

The results of three isolations of the pyrimidine nucleotides are given in Table I.

TABLE I

Expt.	*Specific activity (counts/min/mmole nucleotide)*		
	Uridylic acid	*5-Ribosyluracil phosphate*	*Cytidylic acid*
I	$1.3 \cdot 10^6$	$1.1 \cdot 10^6$	$0.9 \cdot 10^6$
II	$1.4 \cdot 10^6$	$1.5 \cdot 10^6$	$0.9 \cdot 10^6$
III	$1.1 \cdot 10^6$	$1.4 \cdot 10^6$	$0.7 \cdot 10^6$

It is evident from the data in the Table that the incorporation of [^{14}C]orotic acid into uridylic acid and 5-ribosyluracil phosphate is equivalent within the range of experimental error. Cytidylic acid is less heavily labeled, as is commonly found in this kind of experiment[7], probably because of additional steps in its biosynthesis, during which dilution of the isotope by intermediate substances in the metabolic pool in the cell can take place. The equal labeling of uridylic acid and 5-ribosyluracil phosphate implies that the pathway leading to the synthesis of the new nucleotide is as direct as that leading to uridylic acid, or that the new nucleotide is synthesized directly from newly synthesized uridylic acid before it is diluted by the metabolic pool of this material.

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Department of Biochemistry, University of California,
School of Medicine, San Francisco, Calif. (U.S.A.)
JOHN B. HALL
FRANK WORTHINGTON ALLEN

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Effects of some dicarboxylic acids on succinate oxidation *in vitro*

Since the original report on the inhibition of succinoxidase by malonate[1] there has been a continuing interest in the inhibition of this system by other dicarboxylic acids[2]. It was previously noted[3,4] that α-ketoglutarate inhibits the oxidation of succinate *in vitro*, and that aspartate is a competitive inhibitor of succinate oxidation[3]. Evidence presented here indicates that neither α-ketoglutarate nor aspartate is a direct inhibitor of succinate oxidation *in vitro*; the inhibition is mediated by oxaloacetate produced by glutamate–oxalacetate transaminase.

Tissue preparations were made according to standard methods[5]. Succinate oxidation in mitochondrial preparations of human placenta was assayed in a potassium, magnesium phosphate medium[6], pH 7.4; rat-liver mitochondria were assayed by the method of SCHNEIDER AND POTTER[7]. Since the rate of succinate oxidation usually decreases with time, due to the accumulation of oxaloacetate, the results reported represent initial rates from the linear portion of the curves, extrapolated to 1 h.

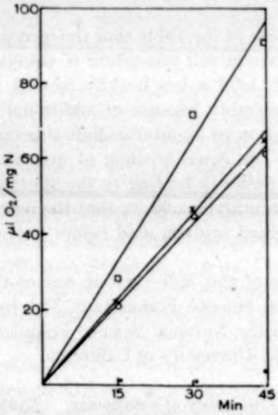

Fig. 1. Effect of α-ketoglutarate and aspartate on succinate oxidation of washed mitochondria from human placenta. Substrates tipped in from side-arm at zero time (30 µmoles per vessel): ×, succinate; ○, succinate + aspartate; □, succinate + α-ketoglutarate; ●, succinate + α-ketoglutarate + aspartate.

In the course of other investigations, it was noticed that the addition of α-ketoglutarate decreased the rate of succinate oxidation in slices of human kidney, prostate, testis, and placenta and in homogenates of human placenta. It was also observed that oxaloacetate, but not α-ketoglutarate, inhibited succinate oxidation by washed mitochondria. Neither α-ketoglutarate nor aspartate added singly inhibited succinate oxidation by washed mitochondria from human placenta (Fig. 1). However, the two together exerted a striking inhibition, suggesting that neither inhibits directly and that the inhibition is the result of the production of oxaloacetate by the transamination of aspartate with α-ketoglutarate.

Abbreviation: DPN, diphosphopyridine nucleotide.

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This mechanism of inhibition presupposes that glutamate–oxalacetate transaminase is present in mitochondria. The need for a quantitative examination of the intracellular distribution of this enzyme was recently pointed out[8]. Its localization in rat liver was studied using the fractionation procedure of WATSON AND SIEKEVITZ[9]; the enzyme was assayed by the spectrophotometric method of GUNSALUS AND STAMER[10]. Approximately one-third of the total glutamate–oxalacetate transaminase activity of the whole homogenate was located in the mitochondria and about one-half appeared in the particle-free supernatant obtained by centrifugation at 105,000 × *g* for 1 h (Table I). Washing the mitochondria as many as eight times failed to remove any more of the activity. Transaminase activity was also present in mitochondria from tissue homogenized in 0.15 *M* KCl buffered with 0.05 *M* phosphate at pH 5.7, 7.4, or 8.0. Treatment with deoxycholate failed to remove the activity from the mitochondrial membrane. Thus, under the conditions of these experiments, the enzymic activity necessary for the mechanism of inhibition proposed appears to be

TABLE I

INTRACELLULAR DISTRIBUTION OF GLUTAMATE–OXALOACETATE TRANSAMINASE IN RAT LIVER

Fraction	*Total activity*[*]	*% total activity*	*Specific activity*[**]
Whole homogenate	1200[***]	100	65
Mitochondria	381	31.8	100
Microsomes	66	5.5	18
Supernatant	550	45.8	69

[*] Results presented as units/g equivalent of liver.

[**] Results presented as units/mg N.

[***] Each value represents the mean of four experiments in units of glutamate–oxalacetate transaminase activity. 1 unit = 1 μmole oxaloacetate produced/h.

TABLE II

EFFECT OF α-KETOGLUTARATE, ASPARTATE, AND GLUTAMATE ON SUCCINATE OXIDATION IN MITOCHONDRIAL PREPARATIONS FROM RAT LIVER

Substrate[*]	*Mitochondria*	*Mitochondria plus DPN*[**]
Succinate	911[***]	280
Succinate, α-ketoglutarate	922	267
Succinate, aspartate	996	309
Succinate, aspartate, α-ketoglutarate	125	25
Succinate, aspartate, α-ketoglutarate, glutamate	558	518
Succinate, glutamate	711	1034
Glutamate	26	359
Number of experiments	6	6
Standard error of mean	42[§]	67

[*] Substrate tipped in from side-arm at zero time as follows: succinate, 50 μmoles per vessel; α-ketoglutarate, 5 μmoles per vessel; aspartate, 5 μmoles per vessel; glutamate, 100 μmoles per vessel.

[**] 1.5 μmoles per vessel.

[***] Results given as mean μl O_2 consumed/mg N/h.

[§] Analysis of variance shows the difference between treatment means to be significant ($P < 0.01$). Standard errors of the mean calculated from the analysis[11].

firmly bound to the mitochondria and should generate enough oxaloacetate to account for the inhibition obtained.

Washed mitochondria from rat liver (Table II) were used to extend the observations made on human tissues. Neither α-ketoglutarate nor aspartate added individually inhibited succinate oxidation, whereas the two added together produced the same striking inhibition seen with similar preparations from human placenta. Glutamate appeared to exert three distinct effects upon succinate oxidation: (a) Without the addition of DPN$^+$, it partially reversed the inhibition of α-ketoglutarate and aspartate added simultaneously; that it was not itself oxidized suggests that this reversal was due at least in part to transamination. (b) In this preparation it also appeared to exert a direct, albeit very weak, inhibitory effect on succinate oxidation. (c) In the presence of added DPN, glutamate was oxidized, and the oxygen consumption in the presence of both succinate and glutamate was far greater than the sum of the two added individually. These observations may explain the apparently conflicting reports on the effect of glutamate on succinate oxidation. Toluene-treated *Escherichia coli* which could oxidize succinate and lactate but not glutamate showed a slight inhibition of succinate oxidation by glutamate[1]. On the other hand, SWINGLE *et al.*[12] reported a stimulation of succinate oxidation by glutamate in whole homogenates of rat heart. It seems likely that the availability of DPN modifies the action which glutamate exerts on succinate oxidation. Under conditions where lactate could undergo oxidation, it similarly increased succinate oxidation[12] whereas pyruvate inhibited it[1].

These experiments suggest that, contrary to previous reports[3,4], neither α-ketoglutarate nor aspartate is a direct inhibitor of succinoxidase. The actual inhibitor is oxaloacetate produced by transamination; the earlier work was carried out in homogenate systems in which the possibility of transamination could not be excluded. Thus, these results are comparable to the recent observations of WOLFE AND BALL[13] who were able to relate the apparent stimulation of succinoxidase by thyroxine to its inhibition of malic dehydrogenase and the subsequent decreased production of oxaloacetate.

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Department of Biological Chemistry, Harvard Medical School, G. GAULL*
and Research Laboratories, Boston Lying-in Hospital, C. A. VILLEE
Boston, Mass. (U.S.A.)

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* Postdoctoral Fellow, National Cancer Institute, U.S. Public Health Service.

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Variations in bacterial ribosomes

Ribosomal particles are an important constituent of all bacterial cells. Particular attention has been focussed on them recently due to their role in protein synthesis, a clear demonstration of this function in bacteria having been made by McQuillen, Roberts and Britten[1]. Study of a large number of ultracentrifugal analyses indicates that the ribosome content of a bacterial cell is characteristic of the physiological state of the cell. The absolute quantity of ribosomes in a cell varies according to the medium in which they are growing, being greater when the cells are growing more rapidly. Furthermore, radical changes in the relative proportions of the various components are discernible as the cell passes from the growing to the resting state and *vice versa*. The ribosomes of resting cells, which have exhausted the supply of the energy source, glucose, are almost entirely of one class whereas exponentially growing cells show peaks corresponding to at least four classes of ribosomes.

A culture of resting cells was prepared by growing *Escherichia coli* B overnight in a glucose (0.8 mg/ml)-salts medium under forced aeration at 37°. In this way the glucose supply had been exhausted some 12 h prior to the commencement of the experiment. After the addition of more glucose, 100-ml samples were taken at intervals and poured upon crushed frozen growth medium. The cells were then harvested and washed three times in 0.01 *M* tris(hydroxymethyl)aminomethane, 0.004 *M* succinic acid and 0.01 *M* magnesium acetate buffer, pH 7.6. Breakage of the cells was effected by means of a modified French pressure cell at 10,000 lbs./in.^{2}. The resulting cell juices were examined in a Spinco Model E ultracentrifuge equipped with Schlieren optics. Sedimentation coefficients were estimated from the plates and corrected for the viscosity change of water to 20°. Fig. 1 shows the progressive changes in the particle pattern during the first 15 min.

The addition of glucose caused appreciable conversion of the large single component into smaller particles during the first 1.5 min. Calculation of the sedimentation coefficients showed that the new particles appearing were the well known ones of 70, 51 and 32 *S*[2,3]. The quantity of the largest component continued to decrease until by 11 min the pattern resembled that of a typical exponential culture. Furthermore, the sedimentation coefficient of the fastest moving component was now 86 *S* and none of the original 100 *S* component remained.

The onset of renewed growth could not be adequately assessed from turbidity measurements but study of the $^{35}SO_4^{=}$ uptake showed that no appreciable protein synthesis occurred during the first 7 min (Fig. 2). The period between 7 and 11 min corresponded to that of an increasing rate of protein synthesis. At the end of this time changes in the ultracentrifuge pattern were almost complete. Thus the recommencement of growth seems to be correlated with the establishment of the normal ratios between the amounts of the various classes of ribosomes.

A similar study of the reverse transition into the resting state suggested that changes in the particle pattern are equally rapid in this direction. Although there is more uncertainty in assessing the time at which the last traces of glucose are swept up by the cells, the ultracentrifugal analysis of two samples taken 5 min apart, just as the turbidity of the culture reached a plateau, gave two very different patterns. While the first was typical of an exponential culture, the second had no components of 85 *S* but consisted mainly of 100 *S* ribosomes.

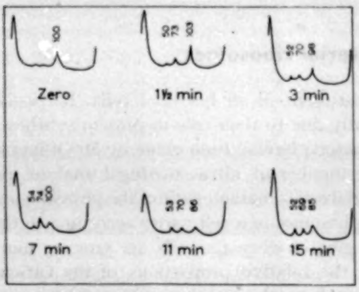

Fig. 1. Sedimentation patterns of cell juice extracted at various times after the addition of glucose. Photographs after 430 sec at 50,740 rev./min with bar angle 30°.

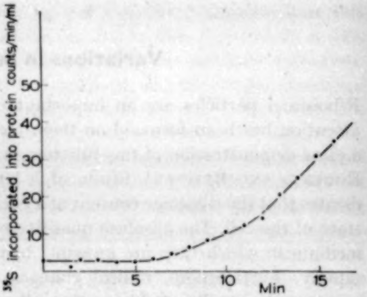

Fig. 2. Uptake of ^{35}S into fraction insoluble in trichloroacetic acid by cells recovering from glucose starvation. ^{35}S and glucose added at time zero.

It is the predominance of the two larger particles of approximately 85 and 70 *S* which characterises exponentially growing cells. Ribosomes with an *S* number as high as 100 appear exclusively in extracts prepared from resting cells. These extracts contain only traces of the slower components. The most important process taking place in the lag before growth of the bacterial cell can recommence may be this fundamental reorganization of the ribosomal material.

The exact relationship between the three large particles having sedimentation coefficients of 70, 85 and 100 *S* is not known. It seems possible, however, that the three objects represent different physical states of the same particle since they may be readily interconverted *in vitro* by changing the Mg^{++} concentration[3]. An analogous transformation may be brought about by an *in vivo* mechanism of the cell. Accordingly 100-*S* ribosomes may be regarded as inert ribonucleoprotein which can rapidly be converted into protein-synthesising machinery.

Department of Terrestrial Magnetism, Carnegie Institution of Washington, Wash. (U.S.A.) B. J. McCarthy

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Preliminary Notes

The reaction of β-lactoglobulin sulfenyl iodide with mercaptans, thiouracil and thiocyanate

We have recently shown that the rapid reaction of iodine with β-lactoglobulin[1,2] at pH 6–7 and at 5° involves solely the conversion of the two sulfhydryl groups of this protein (mol. wt., 37,000) to the sulfenyl iodides:

$$P(-SH)_2 + 2I_2 \longrightarrow P-(SI)_2 + 2H^+ + 2I^-.$$

A similar reaction was demonstrated with ovalbumin. The production of protein sulfenyl iodide had previously been demonstrated by FRAENKEL-CONRAT with the protein of tobacco mosaic virus[3], but it was considered to be a rather special case involving a highly hindered sulfhydryl group. The demonstration of the reaction with ovalbumin and lactoglobulin suggests that it may be more common than has been thought, and has led us to investigate the reactivity of the sulfenyl iodide function in lactoglobulin toward various reagents.

The sulfenyl iodides exhibit a characteristic absorption at 355 mμ, and we have employed spectrophotometric measurements of the change in this absorption to measure the rate of disappearance of sulfenyl iodide following the addition of various reagents. The protein sulfenyl iodide was prepared by adding the desired quantity of 0.1 *N* iodine in 0.2 *N* KI to a solution of $1.5 \cdot 10^{-3}$ mmole of protein in 3 ml 0.15 *M* phosphate buffer, pH 6.1, which was also 0.2 *M* in KI. The temperature was held at 5° throughout. Since I_3^- has an extremely high absorption at 355 mμ, it was necessary in these experiments to add less than the stoichiometric amount, or about 3.5 equivalents, of iodine per mole of lactoglobulin so as to insure the absence of excess I_3^- in the final solution. The absorbancy at 355 mμ of a solution prepared in this fashion is about 0.40. All of the experiments illustrated in Fig. 1 approximated these conditions.

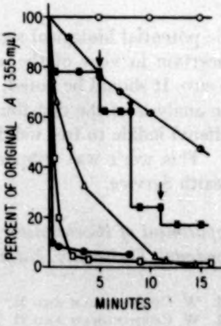

Fig. 1. The disappearance of absorbancy (*A*) at 355 mμ due to the decomposition of β-lactoglobulin sulfenyl iodide following the addition of various reagents: (○), control, no addition; (◐) 25-fold molar excess of cysteine; (■) 5-fold molar excess of SCN^- at zero time, 5-fold excess additional at the first arrow, 50-fold excess additional at the second arrow, and 35-fold excess additional at the last arrow; (△) 50-fold molar excess of 2-mercaptoethanol; (□) 5-fold molar excess of thiouracil; (●) 3-fold molar excess of thiourea. The molar excess noted is that over sulfenyl iodide, based on two of the latter per mole of β-lactoglobulin.

It may be seen that under these experimental conditions the sulfenyl iodide undergoes no appreciable spontaneous decomposition. It has been demonstrated, however, that simple mercaptans will react with sulfenyl iodide to form the mixed disulfide.

$$\mathrm{PSI + RSH \longrightarrow PSSR + H^+ + I^-}$$

Thus FRAENKEL-CONRAT[3] was able to form the mixed disulfide of tobacco-mosaic-virus protein and cysteine, but only after denaturation of the protein. As may be seen in Fig. 1, cysteine in high concentration reacts with the sulfenyl iodide groups of native lactoglobulin. Glutathione is about equally effective. Less complex mercaptans such as 2-mercaptoethylamine and 2-mercaptoethanol react somewhat more rapidly, even at comparable concentrations. In the case of the latter two compounds, the resulting lactoglobulins have been isolated and partially characterized[2]. All the information obtained confirms the formation of the mixed disulfide. For example, neither modified protein contains detectable sulfhydryl groups and the 2-mercaptoethylamine-lactoglobulin derivative yields approximately 2 moles taurine/mole protein following oxidation with performic acid.

A third group of compounds including propylthiouracil, thiouracil, and thiourea were found to be especially reactive toward the sulfenyl iodide. At very low concentrations of these compounds an extremely rapid disappearance of the 355 mμ peak of the sulfenyl iodide occurred, especially with thiourea. Although the products of the reactions have not yet been characterized, the same type reaction presumably occurs as with the mercaptans.

The effectiveness of these reagents led us to examine another substance which antagonizes the normal function of the thyroid, $\mathrm{SCN^-}$. The rate of the reaction of $\mathrm{SCN^-}$ with the protein sulfenyl iodide was found to be essentially instantaneous, but the reaction appears to be a freely reversible equilibrium.

$$\mathrm{P\text{-}SI + SCN^- \rightleftharpoons P\text{-}SSCN + I^-}$$

In several experiments involving the sequential addition of varying quantities of $\mathrm{SCN^-}$ and $\mathrm{I^-}$, the resulting absorbancies at 355 mμ were used to calculate a value for the equilibrium constant of the above reaction.

$$K = \frac{(\mathrm{P\text{-}SSCN})\ (\mathrm{I^-})}{(\mathrm{P\text{-}SI})\ (\mathrm{SCN})} = 10.5$$

The potential biological significance of these results in terms of iodine metabolism is uncertain in view of the absence of evidence for the generation of sulfenyl iodide *in vivo*. It should be noted, however, that isolation procedures currently employed in the analysis of the distribution of organically bound iodine in tissues would convert sulfenyl iodide to free iodide.

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Department of Biochemistry, School of Medicine, LEON W. CUNNINGHAM
Vanderbilt University, Nashville, Tennessee (U.S.A.) B. J. NUENKE

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